

Molecular Identification and Characterisation of Rodent- and Shrew- borne Hantaviruses in South Africa

by
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Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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Summary

Throughout history disease entities have been described which match the description of diseases now known to be caused by hantaviruses; however these viruses were first identified as the aetiologic agent in 1976, the first species named Hantaan virus after the river near which its natural host, the rodent species *Apodemus agrarius*, was captured. Since then numerous species in the *Hantavirus* genus, family *Bunyaviridae*, have been found, with today more than 30 species worldwide being known.

Hantaviruses are hosted by rodents from the *Muridae* and *Cricetidae* families and by shrews (insectivores) in the *Soricidae* family. There are two types of hantavirus disease, Haemorrhagic fever with renal syndrome (HFRS) in the Old World and Hantavirus cardiopulmonary syndrome (HCPS) in the New World. The first two African hantaviruses were identified in 2006 in Guinea, West Africa; Sangassou virus (SANGV) in a rodent, the African wood mouse (*Hylomyscus simus*), and Tanganya virus (TGNV) in Therese's shrew (*Crocidura theresae*).

In this study, rodents and shrews were trapped at localities in the Western Cape and Northern Cape provinces of South Africa, and in the southern regions of Namibia. RNA was extracted from their lungs and screened for hantavirus sequences by RT-PCR, using degenerate primers designed to detect all members of the *Hantavirus* genus.

In addition, an in-house IgG ELISA assay was set up, based on recombinant N antigen from Dobrava virus, DOB-rN, and Puumala virus, PUU-rN. The assay was used to screen patient sera collected in an anonymous convenience serological survey using residual serum samples left over from routine testing at NHLS laboratories in the Western Cape for hantavirus-specific antibodies.

RNA from 576 animal specimens was screened by RT-PCR; no hantavirus genome was detected in any of the specimens. Sera from 161 patients were screened for hantavirus antibodies; 11.18% of the sera were reactive to DOB-rN, 4.97% against PUU-rN and 2.48% against both antigens.

Though no virus was detected in the animals screened, this does not necessarily mean that there are no hantaviruses present in Southern Africa. A previous seroepidemiological survey conducted in South Africa reported on the presence of hantavirus specific antibodies by IFA in two species of rodents trapped in the Western Cape and Northern Cape *Aethomys namquensis* and *Tatera leucogaster*. Our was the second known study in South Africa conducted that determined and proved the presence of hantavirus specific antibodies in humans.

Opsomming

Dwarsdeur die geskiedenis was daar beskrywings van siektes wat ooreenstem met die beskrywing van hantavirus simptome, maar die eerste etiologiese oorsaak van die siekte is eers in 1976 geïdentifiseer en Hantaan virus genoem, vernoem na die rivier waar naby die gasheer, *Apodemus agrarius*, gevang is. Van daar af het die soektog na nuwe hantavirusse intensief gevorder en vandag is daar meer as 30 spesies wêreldwyd wat aan die *Hantavirus* genus, 'n lid van die *Bunyaviridae* familie, behoort.

Knaagdiere van die *Muridae* en *Cricetidae* families, sowel as spitsmuise (insekvreters) in die *Soricidae* familie is gasheer vir hantavirusse. Twee tipes hantavirus siekte is bekend, hemorragiese koors met nier sindroom (HFRS) in die Ou Wêreld en hantavirus kardiopulmonale sindroom in die Nuwe Wêreld. Die eerste twee Afrika hantavirusse is in 2006 in Guinee Wes-Afrika geïdentifiseer; Sangassou virus (SANGV) in 'n knaagdier, die Afrika hout muis (*Hylomyscus simus*) en Tanganya virus (TGNV) in Therese se spitsmuis (*Crocidura theresae*).

In hierdie studie is knaagdiere en spitsmuise op verskeie plekke in die Wes- en Noord-Kaap provinsies, asook die Suide van Namibië, gevang. RNS is onttrek vanuit die longe en hantavirus volgordes is gesoek deur middel RT-PCR deur gebruik te maak van Pan-Hanta primers wat ontwerp is om alle lede van die *Hantavirus* genus op te spoor. 'n Self-ontwerpte IgG ELISA, gebasseer op rekombinante N antigeen van Dobrava virus, DOB-rN en Puumala virus, PUU rN, is opgestel en gebruik om pasiënt serum, verkry in 'n anonieme serologiese opname, te toets; oorblywende serum, na toetse uitgevoer is deur NHLS laboratoriums in die Wes-Kaap, is verkry en getoets vir hantavirus spesifieke teenliggaampies.

RNS van 576 dier monsters is getoets deur middel van RT-PCR en geen hantavirus is in enige van die monsters geïdentifiseer nie. Serum van 161 pasiënte is getoets vir hantavirus teenliggaampies; 11.18% van die serum was reaktief teen DOB-rN, 4.97% teen PUU-rN en 2.48% teen albei antigene.

Alhoewel geen virus in die diere geïdentifiseer is nie, beteken dit nie noodwendig dat geen hantavirusse in Suidelike-Afrika voorkom nie. 'n Vorige sero-epidemiologiese

opname wat in Suid-Afrika gedoen is het die teenwoordigheid van hantavirus spesifieke teenliggaampies in twee knaagdier spesies, *Aethomys namquensis* en *Tatera leucogaster* gevang in die Wes-en Noord-Kaap, gevind. Ons studie is die tweede studie bekend in Suid-Afrika uitgevoer, wat die teenwoordigheid van hantavirus spesifieke teenliggaampies bevind en bewys het.

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The most exciting phrase to hear in science, the one that heralds the most discoveries, is not "Eureka!" (I found it!) But "That's funny..."

Isaac Asimov

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List of Abbreviations

ANDV	Andes virus
ARRV	Ash river virus
bp	base pairs
BSA	Bovine serum albumin
BSL-3	Biosafety level-3
CCHF	Crimean-Congo haemorrhagic fever
CBNV	Cao Bang virus
cDNA	Complementary DNA
cRNA	Copy RNA
CTL	CD8+ Cytotoxic T lymphocytes
DOBV	Dobrava virus
DOB-rN	Dobrava recombinant nucleocapsid antigen
dNTPs	Deoxyribonucleoside triphosphates
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FITC	Fluorescein isothiocyanate
FRNT	Focus reduction neutralisation test
G1/Gc	Glycoprotein 1
G2/Gn	Glycoprotein 2
HCl	Hydrochloric acid
HCPS	Hantavirus cardiopulmonary syndrome
HFRS	Haemorrhagic fever with renal syndrome
H₂SO₄	Sulphuric acid
HRP	Horse radish peroxidase
HTNV	Hantaan virus
ICTV	International committee on taxonomy of viruses
IFA	Immunofluorescence test
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
JMSV	Jemez Springs virus
kDA	kiloDaltons
KHF	Korea Hamorrhagic fever
MgCl₂	Magnesium Chloride
MEM	Minimum essential media

MJNV	Imjin virus
M-MLV	Moloney-murine leukaemia virus
mRNA	Messenger RNA
N	Nucleocapsid protein
NaCl	Sodium chloride
Na₂CO₃	Sodium carbonate
NaOH	Sodium hydroxide
NE	Nephropathia epidemica
NHLS	National health laboratory services
OD₄₅₀	Optical density at 450nm
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PHV	Prospect Hill virus
PMSF	Phenylmethanesulfonylfluoride
PUUV	Puumala virus
PUU-rN	Puumala recombinant nucleocapsid antigen
RdRp	RNA-dependent RNA polymerase
RNA	Ribonucleic acid
RT	Reverse transcription
RT-PCR	Reverse transcription polymerase chain reaction
SANGV	Sangassou virus
SAAV	Saarema virus
SEOV	Seoul virus
SNV	Sin Nombre virus
TAE	Tris acetic acid EDTA
Taq	<i>Thermus aquaticus</i>
TGNV	Tanganya virus
TMB	3, 3',5,5'-Tetremethylbenzidine
TNF-α	Tumor necrosis factor alpha
TPMV	Thottapalayam virus
Tris-CI	tris(hydroxymethyl)amino methane chloride
TULV	Tula virus
vRNA	Viral RNA
YEPD	Yeast Extract Peptone Dextrose

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CHAPTER 1

Introduction

1.1 History of Hantaviruses

There have been reports of diseases likely caused by hantavirus infections throughout history; haemorrhagic fever with renal syndrome-like disease was described in Chinese archives as early as 1000 years ago (Klein & Calisher, 2007). Nephropathia epidemica was described by Swedish scientists in 1934 and several thousands of allied and German troops during World War II were affected by field nephritis (Vapalahti *et al*, 2003; Clement *et al*, 2007). “Songo fever” or epidemic haemorrhagic fever was first described in the 1930s as well; 12 600 cases of disease with fever occurred among Japanese troops during the invasion of northern China (Clement *et al*, 2007).

The hantavirus disease came to the forefront during the Korean war (1950-53) when approximately 3200 cases were reported among the American soldiers (Hart & Bennett. 1999). The causative agent, the Hantaan virus (HTNV) and its host *Apodemus agarius*, was only identified in 1976 (Lee *et al*, 1978). In 1993 another hantaviral disease was reported in the United States of America, when an outbreak of a febrile lung disease with high mortality occurred in the Four Corners region. The causative agent was hitherto unknown member of the genus hantavirus (Enria *et al*, 2001). Subsequent investigations in the area of the outbreak led to the identification of the reservoirs host, the rodent *Peromyscus maniculatus* (Johnson, 2001).

Hantaviruses can be classified into Old World and New World hantaviruses based on geographic distribution and the type of disease they induce in human beings. Hantaviruses are transmitted to human beings from their natural reservoir hosts which are different species of rodents and shrews. Old World hantaviruses are harboured by members of the *Arvicolinae* and *Murinae* subfamilies in Europe, Asia and Africa, whereas the New World viruses are harboured by members of the *Sigmondontinae* and *Neotominae* subfamilies in North and South America (Clement *et al*, 2007; Ramanathan & Jonsson, 2008). Old World hantaviruses cause haemorrhagic fever with renal syndrome (HFRS), which includes Korean and

epidemic haemorrhagic fever and the clinically less severe nephropathia epidemica (NE). In the New World, hantavirus infection results in hantavirus cardiopulmonary syndrome (HCPS) (Vapalahti *et al*, 2003). As many as 150 000 cases of HFRS are reported worldwide, more than half of them in China. More than 21 known hantavirus species are associated with clinical illness, ranging from mild proteinuria to life-threatening haemorrhagic fever and pulmonary oedema (Jonsson *et al*, 2010).

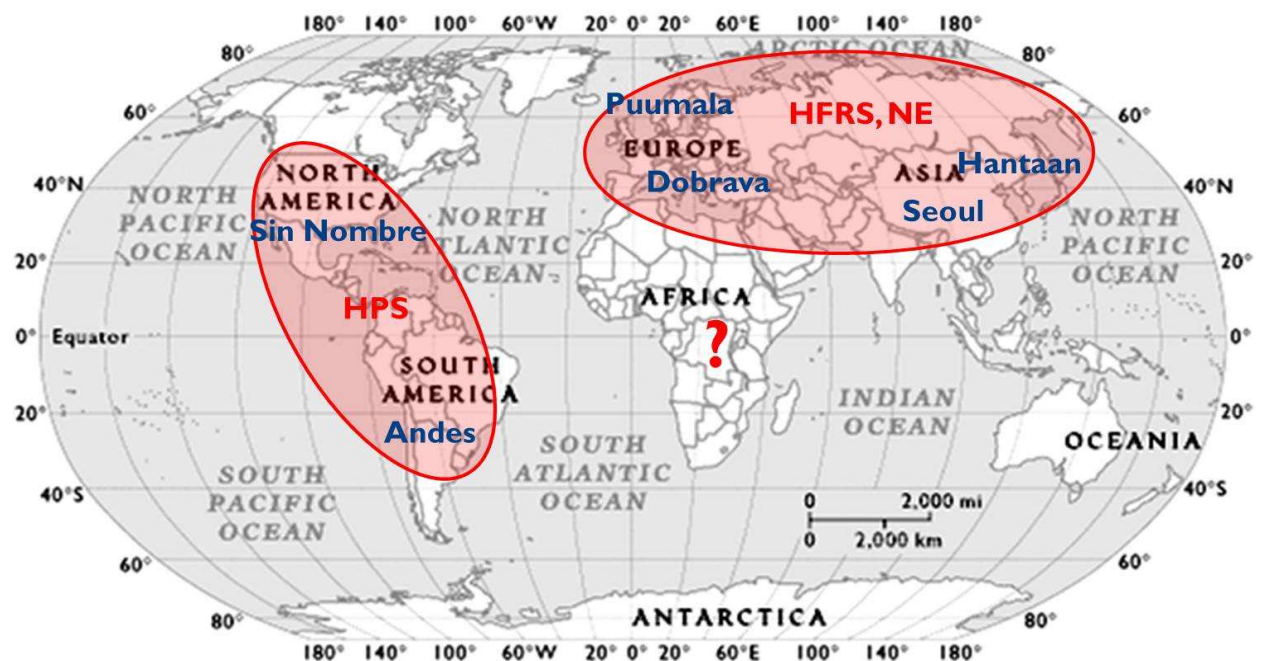


Figure 1.1 Worldwide distribution of hantavirus diseases.

Severe HFRS in Europe is caused by Dobrava virus infection and the milder form of HFRS, NE, is mainly as a result of Puumala virus infection. In Asia, Hantaan virus and Seoul virus infections result in HFRS (though Seoul virus infections have been reported elsewhere in the world due to the ubiquitous distribution of its host *Rattus norvegicus*). In the Americas hantavirus infection results in HCPS disease which is mostly caused by Sin Nombre virus in North and Andes virus in South America [Source: Preiser, 2008].

1.2 Hantaviruses in Africa

The first studies providing evidence for the occurrence of hantavirus infections in Africa was performed by Gonzalez *et al* 1984 in Benin, Burkina Faso, Central African Republic and Gabon (Bi *et al*, 2008). Subsequent serological studies were performed in 1985 in Senegal (Saluzzo *et al*, 1985), in Nigeria (Tomori *et al*, 1986), Djibouti (Rodier *et al*, 1993) and Egypt (Botros *et al*, 2004). In all five studies human sera were tested for hantavirus-specific IgG antibodies. But only one case of HFRS has been reported from Africa, in 1987 in the Central African Republic.

However, the first African hantavirus was only identified in 2006 in Guinea, West Africa. The Sangassou virus (SANGV) was isolated from the African woodmouse *Hylomyscus simus* (Klempa *et al*, 2006). A second hantavirus, the Tanganya virus (TGNV), was identified in a non-rodent insectivore host, Therese's shrew (*Crocidura theresae*), also in Guinea (Klempa *et al*, 2007). The identification of these first hantaviruses in Africa suggests that there may be other hantaviruses in other parts of Africa, too. It is conceivable that these viruses may cause human infection and even disease, which may well have gone undetected so far as hantavirus-induced pathology may be confused with disease caused by other infectious aetiologies such as leptospirosis, rickettsiosis, other viral haemorrhagic fevers, plague, severe pneumonia, sepsis or may simply have remained unrecognized due to poor standards of health care.

1.3 Aims and Objectives

This study was conducted in order to investigate the possible presence of hantaviruses in Southern African small animal reservoirs, i.e. different species of rodents and shrews, by molecular methods, and to characterise any viruses that might thus be identified. The aim of this project is further to use these findings to establish diagnostic assays for the identification of hantaviruses infection in human beings including patients.

Objectives:

- To determine the prevalence of hantaviruses in rodent and shrew reservoirs in Southern Africa
- To identify and characterise novel hantaviruses by molecular and classical virological methods
- To establish serological diagnostic assays for the identification of hantaviruses in human disease cases
- To determine the prevalence of hantavirus antibodies in human beings from different parts of Southern Africa, with particular emphasis on rural areas
- To determine the potential occurrence of hantavirus-induced pathology in human beings in Southern Africa

CHAPTER 2

2 Literature Review

2.1 Natural History of Hantaviruses

The *Hantavirus* genus was formally defined in 1985 (Harper & Meyer, 1999) and currently comprises of more than 30 different species and is the only haemorrhagic fever virus with worldwide distribution including the temperate regions of the Northern hemisphere. Hantaviruses were placed in the *Bunyaviridae* family with four other genera; the *Phlebovirus* and later *Nairovirus*, *Orthobunyavirus* and the plant pathogenic *Tospovirus* genus which includes other human pathogenic viruses such as Rift Valley fever virus and Crimean-Congo haemorrhagic fever (CCHF) virus (Hart & Bennett, 1999; Harper & Meyer, 1999; Weidmann *et al*, 2003).

All other members of the *Bunyaviridae* family are arthropod-borne and are transmitted by vectors such as culicoid flies, mosquitoes, thrips and ticks (Lambert & Lanciotti, 2009), while hantaviruses are transmitted from rodents and insectivores (Weidmann *et al*, 2003; Lambert & Lanciotti, 2009), in which they have co-evolved for millions of years (St Jeor *et al*, 2005; Vaheri *et al*, 2008; Clement *et al*, 2007).

The first hantavirus to be isolated was Thottapalayam virus (TPMV) from the Asian house shrew (*Suncus Murinus*) and was initially classified as an arbovirus, but subsequent investigations showed that the virus belongs in the *Hantavirus* genus by its ultrastructural features and its overall genetic similarities to well characterised rodent-borne hantaviruses (Clement *et al*, 2007; Song *et al*, 2007b). The isolation of TPMV predates the isolation of the HTNV, the prototype virus of the genus by 14 years (Song *et al*, 2007b).

Hantaviruses are divided into 2 main groups based on geographical distribution; Old World in Europe and Asia and New World viruses in the Americas. But there is great divergence in each group and the viruses are further subdivided based on nucleotide and protein sequences (Plyusnin & Morzunov, 2001; Jonsson *et al*, 2010). According to the international committee on virus taxonomy (ICTV) 7% is the minimum protein divergence required for a virus to be considered a species (Lednicky *et al*, 2003). Maes *et al* (2009) suggested the following additions to the current criteria; for group demarcation the amino acid distance must be greater than 24% on the S segment

and greater than 32% on the M segment. In the case of species demarcation, an amino acid distance greater than 10% and 12% on the S segment and M segment respectively.

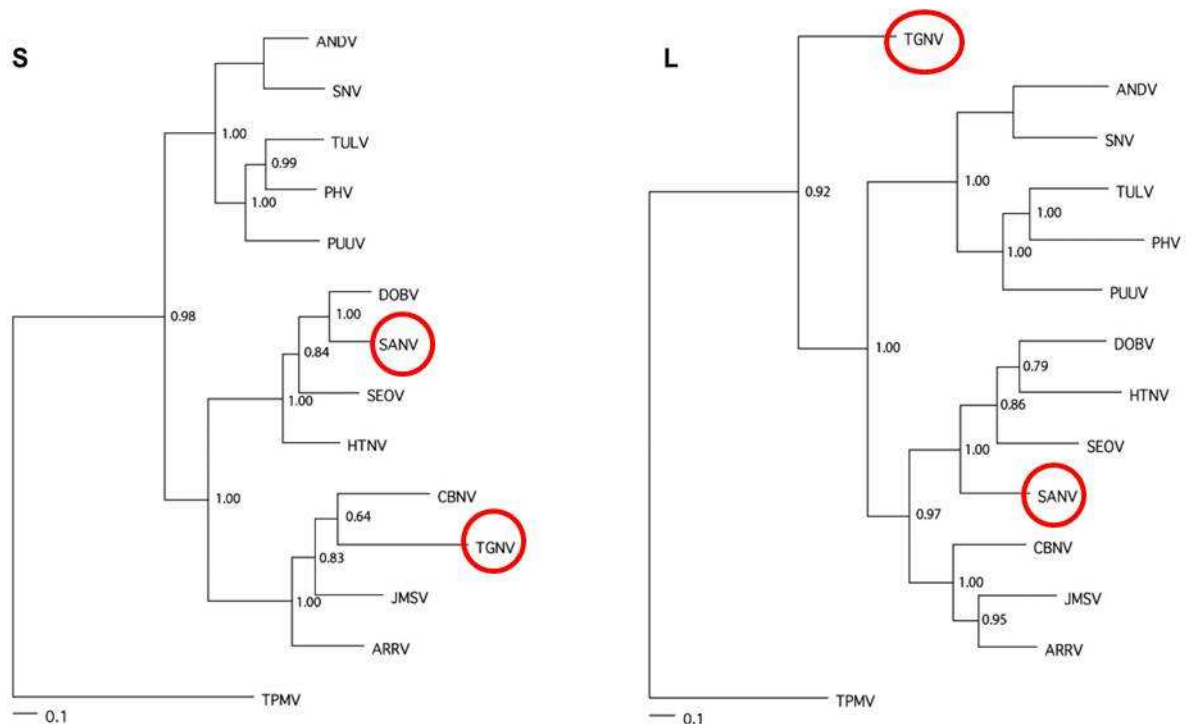


Figure 2.1 Maximum-likelihood phylogenetic consensus trees.

Trees generated based on partial 1048-nucleotide S- (left tree) and 347-nucleotide L- genomic segments (right tree) of shrew-borne viruses; Jemez Springs virus (JMSV) and Ash River virus (ARRV) , Cao Bang virus (CBNV) , Tanganya virus (TGNV) from the Therese shrew (*Crocidura theresae*), and Thottapalayam virus (TPMV) as well as representative *Murinae* rodent-borne hantaviruses; Hantaan virus (HTNV), Sangassou virus (SANV) from the African wood mouse (*Hylomyscus simus*) , Dobrava virus (DOBV), and Seoul virus (SEOV). *Arvicolinae* rodent-borne hantaviruses, Tula virus (TULV), Prospect Hill virus (PHV) and Puumala virus (PUUV) and *Sigmodontinae* and *Neotominae* rodent-borne hantaviruses, including Andes virus (ANDV) and Sin Nombre virus (SNV) [Source: Arai *et al*, 2008].

The figure above is phylogenetic tree constructed from partial L and S segments of some members in the Hantavirus genus. Tree shows that based on the S- segment, viruses are grouped according to reservoir host; *Arvicolinae*-borne viruses cluster together, the same can be concluded for *Murinae*-borne viruses. For the shrew viruses, 4 of the viruses hosted by members of the *Crocidurinae* subfamily cluster together and TPMV. L –segment analysis shows a similar picture for the *Muridae* borne viruses but TGNV does not cluster with CBNV, JMSV and ARRIV as observed for the S –segment.

2.2 Hantavirus Morphology

Hantaviruses are enveloped viruses with a negative sense, single-stranded RNA genome. Like all other members of the *Bunyaviridae* family, the hantavirus genome has three segments (Hart & Bennett, 1999; Vaheri *et al*, 2008). Hantaviruses have the simplest coding strategy in the family; all three segments only encode one protein in the virus complementary sense (McCaughey & Hart, 2000). The large (L), medium (M) and small (S): The large segment (~6500 nt) encodes the RNA-dependent RNA polymerase, the medium segment (~3700 nt) encodes the two envelope glycoproteins: Gn and Gc and the small segment (~1800 nt) encodes the nucleocapsid N protein (Hart & Bennett, 1999; Vaheri *et al*, 2008).

A molecular feature found to distinguish hantaviruses from other members of the *Bunyaviridae* family is the presence of conserved, complementary terminal panhandle nucleotide sequence (AUCAUCAUC) on the L, M and S segments (figure 2.2) (McCaughey & Hart, 2000; Jonsson & Schmaljohn, 2001). It is this characteristic and the absence of cross-reactivity among other members of the family that are the basis for the proposal that led to the establishment of the *Hantavirus* genus in 1985 (Jonsson & Schmaljohn, 2001).

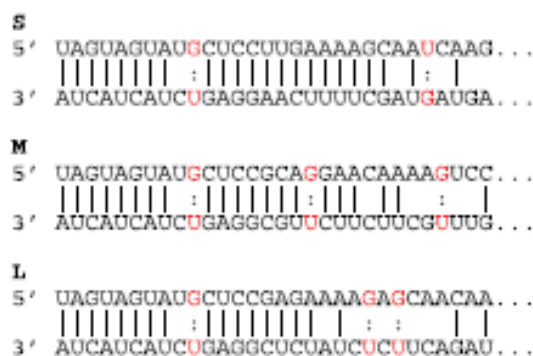


Figure 2.2 The terminal panhandle sequences at the 5'- and 3'- ends of the S, M and L RNA segments. [Source: Kukkonen *et al*, 2005].

By electron microscopy, hantavirus virions are roughly spherical with a diameter that varies from 80 nm to 210 nm (McCaughey & Hart, 2000; Spiropoulou, 2001). A hantavirus virion consists of an internal nucleocapsid arranged in circular coils, it is surrounded by a ~5 nm bi-layered membrane. The surface glycoproteins, Gc and Gn appear as projections that are ~6 nm in length. The virion is composed of >50% protein, 20-30% lipid and 2-7% carbohydrate (McCaughey & Hart, 2000).

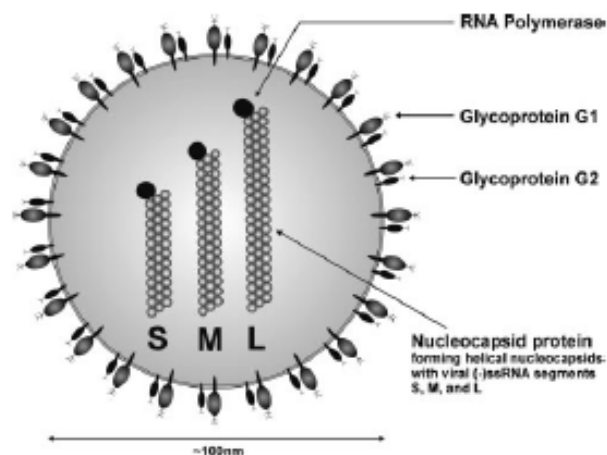


Figure 2.3 Illustration of hantavirus morphology [Source: Muranyi *et al*, 2005]

2.3 Viral Replication and Transcription

The main components of hantavirus replication are the RNA-dependent RNA polymerase (RdRp), the Nucleocapsid protein (N), and the viral genomic and antigenomic RNA templates (Jonsson & Schmaljohn, 2001). The RdRp is responsible for the synthesis of positive strand messenger RNA from the L, M, and S viral RNA segments; it mediates both transcription and replication (Jonsson & Schmaljohn, 2001). Sometime after transcription has been initiated, RdRp initiates virus replication by the synthesis of copy RNA (cRNA) by an unknown mechanism. The newly synthesised cRNA can act as template for the synthesis of viral RNA (vRNA). Two mechanisms have been suggested for the synthesis of vRNA: (1) the UTP initiated genome synthesis pathway or (2) the prime-and-realign mechanism. The vRNA can either serve as a template for mRNA during the early stages of infection or it can be packed into virions in the later stages of infection (St Jeor *et al*, 2005).

Hantavirus transcription and replication takes place in the cytoplasm of the target cell (St Jeor *et al*, 2005). Shortly after virion entry and uncoating, primary transcription is initiated by RdRp, soon after initial transcription viral replication is initiated by RdRp by an unknown mechanism (Jonsson & Schmaljohn, 2001).

Transcription occurs by the prime-and-realign mechanism (Figure 1.3). Process begins with the cleavage of a primer from the 5'end of the host mRNA, all the primers used in transcription must have a G at the 3'terminus (Kaukinen *et al*, 2005). This primer is then aligned a few nucleotides upstream from the 3'end of the viral template and elongated with a few nucleotides, and then realigned so that the G on the host-derived primer is at position -1 and the recently added nucleotides on the mRNA basepair with the viral template (Jonsson & Schmaljohn, 2001). Final elongation then occurs where the entire viral template is transcribed until the RdRp encounters the termination signal. RdRp is responsible for both transcription and replication. The exact mechanism for the switch from primary transcription to replication is not known however the switch most likely occurs once the production of viral proteins such as the N protein (products of primary transcription and translation) reaches a threshold level (Jonsson & Schmaljohn, 2001; Kaukinen *et al*, 2005).

2.4 Entry Mechanism into Target Cells

Virus replication mainly takes place in the macrophages and vascular endothelial cells especially in the lungs and kidneys (Muranyi *et al*, 2005). Pathogenic hantaviruses enter the host cells by attaching to $\alpha_v\beta_3$ integrins on the cell surface and subsequent endocytosis (Gavrilovskaya *et al*, 1998; Muranyi *et al*, 2005).

When hantaviruses infect both the natural reservoir host and humans they infect endothelial cells, macrophages, kidney glomerula and epithelial cells primarily (Jin *et al*, 2002). Early entry of the virus into the target cells involves virus attachment to the β_3 integrin cell surface receptors. The exact mechanism by which hantaviruses attach to the cell surface is unknown however, the pathway by which the virus enters the target cell was determined in experiments using endocytosis inhibitors: the prototype virus of the *Hantavirus* genus, Hantaan virus (HTNV) was used (Mackow & Gavrilovskaya, 2001). Experimentation using the HTNV and endocytosis inhibitors by Jin *et al* concluded that hantaviruses are internalized in to the host cell by clathrin-dependent receptor-mediated endocytosis by showing that inhibition of the clathrin-dependent pathway resulted in the inhibition of infection (Jin *et al*, 2002).

2.5 Hantavirus Infection in Natural Reservoir Host

A variety of small mammals serve as reservoir hosts for hantaviruses. Current evidence suggests those viruses and their hosts have co-evolved, resulting in specific hantaviruses being closely associated with a certain host species. The geographical range of the reservoir host species thus defines the geographic range of the hantavirus: Hantaan virus is carried by *Apodemus agrarius coreae* (field mouse) in Asia (Lee *et al*, 1978) (Figure 2.4). The range of *Apodemus* species includes most of Europe; the Balkans, western Russia, parts of Spain and France as well as south Scandinavia and extends into Asia China, Korea, Japan and Mongolia. *Apodemus agrarius* present in Asia and Europe harbours Hantaan and Dobrava virus (Muranyi *et al*, 2005). Puumala virus is carried by *Myodes glareolus* (bank voles) in Europe and its range is restricted to most of Western Europe and Scandinavia as well as the British Isles and Ireland (Muranyi *et al*, 2005).

The Seoul virus is the only known hantavirus with a worldwide range because it's hosts *Rattus* species are found worldwide (Mackow & Gavrilovskaya, 2001; Jonsson *et al*, 2010). Sin Nombre and New York viruses are by *Peromyscus* species (*Neotominae* subfamily) and found exclusively in America (Clement *et al*, 2007).



Above: *Apodemus agrarius coreae*



Above: *Hylomyscus simus*



Left: *Peromyscus maniculatus*

Figure 2.4 Selected hantavirus reservoir hosts.

Apodemus agrarius coreae is the host for Hantaan virus (HTNV), the prototype virus of the genus. *Peromyscus maniculatus* is the host of Sin Nombre virus (SNV) and *Hylomyscus simus* is the host for the first African hantavirus that has been identified, Sangassou virus (SANGV). [Sources: discoverlife.org, animalpicturesarchive.com & sflorg.com/sciencenews]

Table 2.1 Selected hantavirus species, their main reservoir host and human disease that they cause. [Modified from Schönrich *et al*, 2008]

	Order	Family	Subfamily	Species	Virus	Human disease
Old World viruses	Rodentia	Muridae	Murinae	<i>Apodemus agrarius</i>	Hantaan	HFRS
				<i>A. flavicollis</i> , <i>A. agrarius</i>	Dobrava	HFRS
				<i>Rattus rattus</i> , <i>Rattus norvegicus</i>	Seoul	HFRS
				<i>Hylomyscus (alleni) simus</i>	Sangassou	
				<i>Myodes glareolus</i>	Puumala	NE
			Arvicolinae	<i>Microtus arvalis</i> , <i>M. agrestis</i>	Tula	HFRS
		Soricidae	Crocidae	<i>Crocidae theresae</i>	Tanganya	Unknown
				<i>Suncus murinus</i>	Thottapalayam	Unknown
			Soricinae	<i>Sorex araneus</i>	Seewis	Unknown
				<i>Anourosorex squamipes</i>	Cao Bang	Unknown
New World viruses	Rodentia	Muridae	Arvicolinae	<i>Microtus pennsylvanicus</i>	Prospect Hill	HCPS
				<i>M. ochrogaster</i>	Isla vista	HCPS
			Neotominae	<i>Peromyscus maniculatus</i>	Sin Nombre	HCPS
				<i>P. leucopus</i>	New York	HCPS
			Sigmodontinae	<i>Oligoryzomys longicaudatus</i>	Andes	HCPS
				<i>other Oligoryzomys sp.</i>		
				<i>Oryzomys palustris</i>	Bayou	HCPS
		Soricidae	Soricinae	<i>Sorex cinereus</i>	Ash River	Unknown
				<i>S. monticolus</i>	Jemez Springs	Unknown
				<i>Blarina brevicauda</i>	Camp Ripley	Unknown

The distribution of hantavirus infections in a host species population is affected by behavioural patterns. Males are more frequently infected than females because they engage more frequently in aggressive behaviour which results in wounding and thus the transmission of infection. The incidence of infection also differs between juvenile and adult males. The incidence and severity of wounding is much higher in adult males and thus the incidence of hantavirus infection as well (Hinson *et al*, 2004). A study in bank voles found that adults with most wounds at the end of the breeding season (autumn) would be more likely to be infected than the non-wounded animals (Escutenaire *et al*, 2002).

Longitudinal studies of Norway rats infected with Seoul virus and deer mice infected with Sin Nombre virus showed that in a population of rodents, age determines who is infected with the virus and wounding during fights is the primary means by which infection is acquired or transmitted (Botten *et al*, 2003; Hinson *et al*, 2004; Easterbrook & Klein, 2008). Susceptibility to viral infection differs between males and females. Males are more susceptible to infection because females mount a higher immune response than males. Observation of Norway rats infected with the Seoul virus, have shown that males shed the virus for longer periods of time and via more routes than the females. The viral load in the target organs is also much higher in the males (Klein *et al*, 2002; Hannah *et al*, 2008). Transmission among rodents also occurs by inhalation of aerosol of excrement and urine that contain infectious viral particles (Easterbrook & Klein, 2008).

Rodents infected with persistent hantavirus infection are not put at any survival disadvantage. They do not suffer any detrimental effect on reproductive fitness (McCaughey & Hart, 2000). The mechanisms which support hantavirus persistence in reservoir hosts have not been clearly defined. One major hypothesis suggests that hantaviruses maintain persistence in the host by suppressing the immune responses required to clear infection (Easterbrook *et al*, 2007). Another possible persistence mechanism is replication in immune cells such as monocytes, macrophages and T lymphocytes. Hantaviruses also probably avoid detection by the host immune system by regulating viral replication and viral protein expression (Meyer & Schmaljohn, 2000).

Human epidemics can be predicted by the cyclic fluctuations on reservoirs populations; a recent study showed a direct correlation between an increase in bank

vole population in previous spring and human cases in the autumn of Nephropathia epidemica (Kallio *et al*, 2009). The outbreak of epidemics in humans is closely correlated to increased reservoir population. In central Europe the increase in rodent populations occurs due to mast years. A mast year is a year when there's increased abundance in tree nuts on the forest floors, a food source for rodents. This increase in food source can be attributed to high summer temperatures 2 years prior and high autumn temperature in the previous year; these high temperatures favour flower and seed development which then results increased nut production. Increased food source during mast years results in increased rodent population, enough to maintain transmission of the virus within the population and to humans (Jonsson *et al*, 2010). The similar phenomenon is observed in North America, where increased precipitation leads to increased food resources for the rodents and an increase in the reservoir population (Engelthaler *et al*, 1999).

2.6 Epidemiology

Hantaviruses that cause human infection and disease are hosted by rodents. There is no solid evidence that insectivore-hosted viruses cause disease in humans. Human beings are primarily infected by aerosolised rodent faeces, urine and saliva (Vaheri, 2008), but humans have also been infected through contact with open wounds and rodent bites (Hammerbeck *et al*, 2008).

The discovery of HTNV as the causative agent of Korea haemorrhagic fever (KHF) led to epidemiological studies in rodents and humans (Jonsson *et al*, 2010). Infections in humans are not age- or gender dependent. All exposed individuals have the same risk of infection and disease. However, epidemiological studies show that most cases of both HFRS and HCPS occur in working-age males. This phenomenon is most likely related to occupational exposure: agricultural or forest work where individuals are most likely to come into contact with infected animals (Hammerbeck *et al*, 2009).

There are an estimated 150 000 to 200 000 HFRS cases per year; 100 000 of which occur in China (Maes *et al*, 2004; Bi *et al*, 2008). Clinical cases have been reported in other Asian countries such as Taiwan and South Korea (Bi *et al*, 2008). In Europe, HFRS is caused by DOBV, PUUV and TULV. DOBV predominantly occurs in the Balkans and Eastern Europe, and it is the most virulent European virus

with a mortality rate of up to 12% (Maes *et al*, 2004). PUUV has the widest geographical range in Europe, human infection results in Nephropathia epidemica, a milder form of HFRS with a case fatality rate of 0.1% (Clement *et al*, 2007). The first reported outbreak of hantavirus disease in the Americas was in 1993; disease outbreak occurred in the four corners region in the USA. The causative agent was Sin Nombre virus (SNV) hosted by *Peromyscus maniculatus*. The first HCPS case in South America was reported in Argentina caused by the Andes virus, in 1995 with 29 cases reported (Bi *et al*, 2008). Andes virus is the only hantavirus for which person-to-person transmission was reported (Martinez *et al*, 2005).

Serological evidence of hantavirus infections (in both humans and rodents) in Africa was found in 1984 by Gonzalez *et al*. serological surveys were done in Central African Republic, Gabon, Benin and Burkina Faso. Evidence of human hantavirus infections was also found in Egypt, Nigeria, Djibouti and Senegal (Bi *et al*, 2008). Only one case was reported in Africa; in the Central African Republic in 1987 (Coulaud *et al*, 1987). The first African hantavirus was identified in Guinea, west Africa. It was named Sangassou (SANGV) after the village near which the host was trapped (Klempa *et al*, 2006). A follow-up study was done in Guinea in 2009. The prevalence of hantavirus antibodies was 1.2% and Sangassou specific antibodies were found in 2 patients (Klempa *et al*, 2010).

2.7 Hantavirus Disease

Hantaviruses cause disease in humans but not in their animal hosts. No hantavirus disease has been reported in other animals, but experimental infection of hamster has been done to study the HCPS disease progression (Milazzo *et al*, 2002). Both the pathogenic and non-pathogenic viruses have the same tissue tropism. They mainly replicate in the endothelial cells and macrophages (Maes *et al*, 2004). In both HFRS and HCPS viraemia is thought to occur subsequent to infection of alveolar macrophages, resulting in the infection of kidney and lung endothelial cells (Maes *et al*, 2004). There are disease features common to both HFRS and HCPS; increased vascular permeability (which results in hypotension, hemoconcentration and vasodilation), increased TNF- α production, acute thrombocytopenia, CD8+ T lymphocyte activation, and leukocytosis (Schönrich *et al*, 2008; Vaheri, 2008). The

primary target in HFRS is the kidneys but pulmonary involvement has been reported in patients and the same is true for HCPS where the lungs are the primary target and renal involvement occurs as well (Vaheri, 2008).

2.7.1 Haemorrhagic Fever with Renal Syndrome

HFRS disease presentation ranges from febrile disease to fulminant haemorrhagic shock and death. The incubation period for HFRS ranges from 10 days to 6 weeks and the average is 3 weeks. The severity of disease and clinical pattern vary from subclinical to fatal. HFRS caused by HTNV, Amur virus and DOBV is more severe, while SEOV is more moderate and PUUV causes mild Nephropathia epidemica (Jonsson *et al*, 2010). Febrile phase begins abruptly accompanied by headache and myalgia. This phase lasts 3 to 7 days and 11 to 40% of patients progress to the hypotensive phase (McCaughey & Hart, 2000; Jonsson *et al*, 2010). During this phase patients experience thirst, restlessness, nausea and vomiting which lasts for hours or days. Thrombocytopenia, petechial haemorrhages, proteinuria, conjunctival injection and acute myopia may occur (Jonsson *et al*, 2010). The decrease in blood pressure due to vascular leakage may result in fatal shock syndrome. After 3 to 7 days, the oliguric phase begins and it is characterised by decrease in kidney function resulting in oliguria (or anuria), proteinuria, abnormal urinary sediment, including microscopic hematuria, and azoturia (Schönrich *et al*, 2008). Blood pressure may return to normal though patients are at risk of developing hypertension and pulmonary oedema (McCaughey & Hart, 2000; Jonsson *et al*, 2010). Dialysis is required for 20% of SEOV patients and 40% of HTNV patients (Jonsson *et al*, 2010). The oliguric phase lasts for 3 to 7 days. Urinary output increases during the diuresis phase and patients can pass up to several litres of urine per day for several weeks (McCaughey & Hart, 2000). Convalescence is prolonged and it is several months before the patients fully recover. The mortality rate for HFRS ranges from 5% to 15% for HTNV and DOBV (Schönrich *et al*, 2008).

2.7.2 Nephropathia Epidemica

Nephropathia Epidemica (NE) is a milder form of HFRS. It mainly occurs in Scandinavia and Central Europe, and is mainly caused by PUUV or SAAV. The incubation period before onset of symptoms ranges from 1 to 5 weeks (Pettersson *et al*, 2008). More than 90% of NE cases are asymptomatic and case fatality ranges from 0.1-1% (Vaheiri, 2008; Hammerbeck *et al*, 2009). NE patients usually present with high fever, headache, backache, and abdominal pain (Muranyi *et al*, 2005). NE may also present with conjunctival haemorrhages, petechiae and truncal rash 3 to 4 days after onset of symptoms. Approximately 1% of NE patients develop severe neurological complications such as bladder paralysis or seizures (Muranyi *et al*, 2005; Hammerbeck *et al*, 2009). A 3 day oliguric phase is followed by polyuria and convalescence extends over several weeks, but sequelae are rare (Hammerbeck *et al*, 2009).

2.7.3 Hantavirus Cardiopulmonary Syndrome

Hantavirus cardiopulmonary syndrome (HCPS) initially presents with a prodromal, febrile phase that lasts 3-5 days. Patients initially present with flu-like nonspecific symptoms such as fever, myalgia, malaise, headache, abdominal pain, nausea, vomiting, and sometimes a transient skin rash and conjunctival suffusion (Muranyi *et al*, 2005). The start of the cardiopulmonary phase which is marked by the non-productive cough, shortness of breath, and tachypnea are as a result of pulmonary oedema, respiratory failure and cardiogenic shock (Hammerbeck *et al*, 2009). The mortality rate during this phase is 50% for SNV and ANDV, and patients who survive enter the diuretic phase; rapid clearance of the pulmonary oedema and diuresis occurs. The convalescent phase that follows is marked by fatigue and abnormal pulmonary function but full recovery usually occurs (Hammerbeck *et al*, 2009).

2.8 Hantavirus Pathogenesis

No main factor has been identified to explain the pathogenesis of HFRS and HCPS (Gavrilovskaya *et al*, 1999; St Jeor *et al*, 2005). Evidence from infected patients and experimentally infected hamsters suggests that hantaviruses primarily target endothelial cells (Hammerbeck *et al*, 2009). Studies in which Vero E6 cells, human umbilical cord vein endothelial cells and CHO cells were transfected with recombinant integrins indicate that $\beta 3$ integrins facilitate the entry of pathogenic

HCPS-associated hantaviruses (Gavrilovskaya *et al*, 1998). Similar experiments were also performed to determine the receptors that mediate cellular entry of HFRS-causing hantaviruses, in which the ability of ligands and antibodies to inhibit infection of endothelial cells, vero E6 cells and β 3-integrin transfected CHO cells by Puumala, Seoul Hantaan and Prospect Hill viruses. Results showed that Vitronectin was the most effective inhibitor of PUU and SEO infection but was less effective against HTN and had no effect on PH. Results from these experiments suggest that β 3-integrins mediate entry of HFRS-causing hantaviruses, it also suggests that additional cell surface interactions contribute to hantavirus entry as uninhibitable hantavirus infectivity was observed (Gavrilovskaya *et al*, 1999). In contrast, the entry of non-pathogenic PHV is not mediated by β 3-integrins; PHV infectivity was inhibited by α 5-antibodies and β 1-antibodies which suggest that PH cellular entry is mediated by α 5 β 1-integrin receptors (Gavrilovskaya *et al*, 1999; 2002).

The course of infection and severity of hantavirus disease is determined by the degree of increased permeability of the infected endothelial cells. The mechanism by which pathogenic hantaviruses induces capillary leakage during the acute phase of both HFRS and HPS is not yet fully understood (Muranyi *et al*, 2005). However, experimental results from Gavrilovskaya *et al* suggest that hantavirus infection inhibits β 3-integrin-directed endothelial cell migration (Gavrilovskaya *et al*, 2002). The $\alpha_v\beta_3$ -integrin receptor is a heterodimeric receptor composed of α and β subunits that are responsible for cell-to-cell adhesion, cell migration, Ca^{2+} channels (regulation of arteriolar smooth muscle), extracellular matrix protein recognition and platelet aggregation (Gavrilovskaya *et al*, 1999; Gavrilovskaya *et al*, 2002; Maes *et al*, 2004). The $\alpha_v\beta_3$ -integrins are abundant surface receptors of endothelial cells, platelets and macrophages and the interaction of between the $\alpha_v\beta_3$ -integrins and hantaviruses provides the potential for dysregulation of normal endothelial cell functions and contributing to increased vascular permeability observed in hantavirus diseases (Gavrilovskaya *et al*, 2002).

The exact mechanism by which hantavirus infection results in vascular permeability is unknown as *In vitro* experiments show that hantavirus infection does not cause visible cytopathic effect in target cells, nor does it cause changes in the permeability of the endothelial layer, and investigation of postmortem tissue from infected patients does not show visible endothelium damage (St Jeor *et al*, 2005).

Experimental evidence from *in vitro* studies by Gavrilovskaya *et al*, suggests that pathogenic HCPS- and HFRS-causing hantaviruses both enter the target cells via β 3-integrin receptors (Gavrilovskaya *et al*, 1998; Hammerbeck *et al*, 2009). This suggests that pathogenesis is multifactorial and is attributed to the direct effect of the virus on the host cell, the production of TNF- α by infected macrophages and cytotoxic effect of hantavirus specific CD8+ T lymphocytes (CTLs) on infected cells (St Jeor *et al*, 2005; Terajima *et al*, 2007; Jonsson *et al*, 2010). *In vivo* and *in vitro* studies have showed that there are increased cytokine levels during hantavirus infection (Vapalahti *et al*, 2001; St Jeor *et al*, 2005). TNF- α reach highest level in plasma of HFRS-infected patients 3 to 5 days after the onset of disease, and lung and kidney biopsies showed a high number of TNF- α positive cells (St Jeor *et al*, 2005). Experimental evidence suggests that overproduction TNF- α may result in severe systemic toxicity; additionally it may act as a mediator of septic shock (Vapalahti *et al*, 2001) as well as capillary leakage. Experimental data from Hayasaka *et al* suggests that SNV-specific CTLs contribute to the capillary leakage that is observed in HCPS disease (Terajima *et al*, 2007). The role of CTLs in increasing capillary permeability was illustrated using the transwell permeability assay where an SNV-infected endothelial cell line was exposed to SNV-specific CTLs. CTL induced permeability is probably due to production of cytokines such as TNF- α by CTLs rather than cell lysis as autopsies from fatal HCPS cases show no visible damage to endothelial cells (Hayasaka *et al*, 2007). Higher frequency of CTLs were observed in hospitalized patients with clinically severe SNV-HCPS disease requiring mechanical ventilation compared to those who had mild disease (Kilpatrick *et al*, 2004).

Our understanding of hantavirus pathogenesis is limited by the absence of disease in reservoir hosts and the lack of an animal model. The analysis of cellular differences that might be associated with the viral phenotypes that determine pathogenicity and non-pathogenicity is hampered by the lack of cell lines from hosts (Gavrilovskaya *et al*, 1998; 2002; Mackow & Gavrilovskaya, 2001).

2.9 Immune Response

Innate response occurs in response to hantavirus infection of the host cell. Expression of various type I interferon and other proinflammatory cytokines as well as the activation of interferon inducible genes is required for the induction of cellular viral resistance and activation of innate immune cells such as Natural killer cells (Schönrich *et al*, 2008). Studies have shown the expression of MxA protein is delayed in cells infected with pathogenic virus compared to the cells infected with non-pathogenic virus. The same phenomenon was observed for the upregulation of HLA class I molecules. Other antiviral mechanisms such as the classical and alternative route of the complement system are also activated during infection (Muranyi *et al*, 2005).

All types of Immunoglobulins are expressed during HFRS- and HCPS- causing hantavirus infection. The main target of antigen in the N protein but antibody titres against the glycoproteins G1 and G2 are also produced (Kanerva *et al*, 1998; Muranyi *et al*, 2005). IgA is detected during the acute phase of disease; however the anti-viral protection mechanism in human infection is unknown. Studies in rats have shown that maternal IgA protect the infants from lethal doses of SEOV (St Jeor *et al*, 2005). IgE serum levels are increased during infection and it is hypothesized that IgE plays a role in hantavirus pathophysiology by activating the secretion of IL-1 β and TNF- α which influence permeability of the infected endothelium (Muranyi *et al*, 2005). IgM antibodies have been observed against all three viral structural proteins and it is detected during the acute phase of infection and levels decline during the convalescent phase coinciding with the increased levels of IgG (Kanerva *et al*, 1998; St Jeor *et al*, 2005). IgG titre is higher for G1 and G2 than N protein (St Jeor *et al*, 2005).

Cytotoxic CD8⁺ T lymphocytes (CTLs) are the predominant lymphocyte present during a hantavirus infection (Muranyi *et al*, 2005) as they have been detected blood samples obtained from infected animals as well as patients in convalescence (Kanerva *et al*, 1998). CTLs play an important role in the clearance of the infection and in pathogenesis of HFRS and HCPS (Muranyi *et al*, 2005). Studies by Kilpatrick *et al* show that the severity of HCPS disease correlates with the number of CTLs. Using flow cytometry, they illustrated the relationship between the frequency of

CD8+ cells (as well as increased presence of TNF- α , IFN- γ and IL-2 producing cells in the lungs of HCPS patients) and severity of disease (Kilpatrick *et al*, 2004).

CTL epitopes have been identified for the three viral structural proteins; Glycoprotein 1 (Gc), Glycoprotein 2 (Gn) and Nucleoprotein (N) which is the major antigen responsible for the activation of the T cell response during infection (Muranyi *et al*, 2005; St Jeor *et al*, 2005).

2.10 Diagnosis of Hantavirus Infection

The diagnosis of hantavirus infection in human beings is based on clinical and epidemiological information as well as laboratory tests. A definite diagnosis cannot be based solely on clinical findings especially in cases where disease is mild to moderate (Bi *et al*, 2008). Testing should be performed on samples from patients with fever of unknown origin, lumbago, renal failure, respiratory distress and recent outdoor activities during which there was possible exposure to rodents and shrews or their excreta (Muranyi *et al*, 2005).

Diagnosis of hantavirus infection is mainly based on serological testing because viremia is short-lived and viral RNA cannot consistently be detected in human blood and urine specimens. PCR has been used successfully in detecting PUU RNA in some patient specimens, but the short duration or absence of viraemia during the acute phase of infection means that other methods must be used for patient diagnosis (Sjölander & Lundkvist, 1999).

Serology is ideal because high levels of virus-specific antibodies can be detected at the onset of disease (Vapalahti *et al*, 2001; Bi *et al*, 2008); the highest antibody titres are observed between day 8 and 25 (Muranyi *et al*, 2005). One of the first serological tests in Europe and Asia was indirect immunofluorescence assay (IFA) using hantavirus-infected cells (fixed on microscope slides) as antigen (Jonsson *et al*, 2010). However using infected cells in a diagnostic assay has its disadvantages as BSL-3 conditions are required for cell culture infections (Bi *et al*, 2008). Thus most serological assays consist of recombinant hantavirus proteins N, Gn and Gc protein as antigens but most assays are based on the recombinant N protein as the N protein is the most abundant viral protein which also induces a strong humoral response in both humans and rodents (Jonsson *et al*, 2010). Other assays such as

Enzyme immunoassay and western blot are used for diagnosis but IgG and IgM indirect enzyme-linked immunosorbent assay (ELISA) is the most common. The detection of IgM is important for the diagnosis of acute infection especially in areas in which hantavirus infection is endemic and there is a high prevalence of IgG in the population due to previous infection (Bi *et al*, 2008).

Although the above assays are ideal for in determining whether a patient is infected with hantavirus, none of these tests can determine which hantavirus is responsible for the infection as significant humoral cross-reaction occurs between the different hantaviruses (Bi *et al*, 2008; Jonsson *et al*, 2010). The infecting hantavirus can only be serotyped by the focus reduction neutralisation test (FRNT) which is the gold standard for hantavirus testing (Vapalahti *et al*, 2001; Bi *et al*, 2008). FRNT can detect and measure neutralizing antibodies by comparing serum titres to the relevant hantaviruses (Vapalahti *et al*, 2001; Jonsson *et al*, 2010) and though it is capable of distinguishing hantaviruses with serum from experimentally infected rodents it was found to be less specific when serum from acute phase patients was tested (Jonsson *et al*, 2010) other drawbacks of FRNT is that it (i) is time-consuming and labour-intensive, and (ii) must be carried out under BSL-3 conditions because infected cell cultures are used in the assay (Vapalahti *et al*, 2001; Bi *et al*, 2008).

The identification of the hantavirus responsible for infection can also be achieved by molecular methods such as virus-specific RT-PCR (Bi *et al*, 2008; Jonsson *et al*, 2010), however virus-specific assays can only be used if the suspected agent is known beforehand. Therefore, RT-PCR using universal primers to recognise most or all members of the genus may be used and the suspected species of virus is genotyped by sequencing. RT-PCR can be used detect RNA from fresh/frozen tissue, blood and serum (Bi *et al*, 2008). RT-PCR can be useful as a rapid method for hantavirus detection in HCPS cases where disease is fast-evolving; patients can evolve from acute illness to severe pneumonia and respiratory disease in 12 to 24 hours (Jonsson *et al*, 2010). One-step assays with proven specificity, sensitivity and reproducibility have been developed for hantavirus detection based on real-time RT-PCR and results can be obtained within 24 hours it must be note however that PCR is only useful in the early stages of infection when patients are viremic (Bi *et al*, 2008). Virus isolation from human samples is very rare; it is therefore not considered an option in the diagnosis of human hantavirus infection (Bi *et al*, 2008).

2.11 Prevention and Treatment of Human Hantavirus Infections

Treatment for hantavirus infection in humans is supportive to keep symptoms in control as no effective antiviral drug has been developed as yet (Muranyi *et al*, 2005; St Jeor *et al*, 2005). Effective treatment is achieved by careful fluid management, control of electrolyte balance and hemodynamic monitoring (St jeor *et al*, 2005). Patients with HFRS and HCPS are supervised in an emergency medicine or intensive care unit until the virus is cleared and convalescence begins (Muranyi *et al*, 2005).

There are no antiviral drugs or immunotherapeutic agents that are FDA approved for the treatment of hantavirus infection (Hammerbeck *et al*, 2009). Evidence from In-vitro and animal studies suggests that ribavirin has the ability to inhibit hantavirus replication (McCaughey & Hart, 2000). Ribavirin has been used in the treatment of HFRS patients in China. Ribavirin was able to reduce viral titres, increase survival rates and reducing the severity of disease in patients (Hammerbeck *et al*, 2009; Jonsson *et al*, 2010). However, ribavirin trials on HCPS patients have no effect on the outcome of disease. The negative results observed might be because patients were treated after the onset of the cardiopulmonary phase where as HFRS patients were treated before the onset of renal complications. This evidence suggests that the efficacy of ribavirin is dependent on the phase of infection and severity of disease at the time of administration (Hammerbeck *et al*, 2009). Studies in China have shown that α -interferon has no effect on mortality or the clinical course of HFRS (McCaughey & Hart, 2000).

A variety of hantavirus vaccines has been developed using to main approaches; inactivated virus and subunit molecular virus vaccines (McCaughey & Hart, 2000). Inactivated virus vaccines include viruses replicated in rodent organs or cell culture, the virus is then chemically inactivated with 0.05% of formalin or β -propiolactone and then combined with an adjuvant (Hammerbeck *et al*, 2009). Inactivated-virus vaccines based on HTNV, PUUV and SEOV have been produced and tested in humans in China and Korea (Table 2.2).

Table 2.2 Inactivated virus vaccines currently authorised for use in China and Korea.
[Source: Bi *et al*, 2008].

Virus strains	Generated in	Vaccination Programme	Protection	Country
HTNV	MGKC ⁺	3 basic doses + 1 booster	>90%	China
SEOV	GHKC ⁺⁺	2 basic doses + 1 booster	>95%	China
HTNV	SMB ⁺⁺⁺	3 basic doses + 1 booster	>90%	China
HTN/SEO	MGKC	2 basic doses + 1 booster	100%	China
HTNV	SMB	2 basic doses + 1 booster	75-100%	Korea

+ MGKC: Mongolian gerbil kidney cell;

++ GHKC: Gold hamster kidney cell;

+++SMB: suckling mouse brain.

There are four types of subunit molecular vaccines: protein-based, DNA, virus-like particles, live-virus vectored as well as packaged replicons. Protein vaccines are produced by recombinant baculovirus, *E. coli*, yeast and transgenic plants. In most of the studies performed in animal models, humoral and cellular immune responses were observed. The study by Schmaljohn *et al* in hamsters, is the only one published that investigated the immunogenicity and protective efficacy of purified recombinant glycoproteins in subunit vaccines and found that the best results were observed in a vaccine that includes both Gc and Gn. In another study by Bharadwaj *et al* in 2002, results indicated that the highest level of protection was elicited with Gn peptides. Of all the known subunit molecular vaccines only DNA vaccines have been tested in non-human primates; Custer *et al* and Hooper *et al* tested a HCPS DNA plasmid vaccine based on ANDV M gene segment. High levels of neutralizing antibodies were observed in macaques and rabbits (Hammerbeck *et al*, 2008).

The most effective means of control of hantavirus disease is to minimise human exposure to infected rodents and their excrement. Monitoring hantavirus prevalence in local rodent populations may give some warning to expected increase in the incidence of human cases. Exposure can be minimised by following measures such as rodent-proofing of homes and workplaces (in agriculture and forestry), minimize food available for rodents, adequate disposal of dead rodents (McCaughey *et al*, 2000; Bi *et al*, 2008).

CHAPTER 3

3 Materials and Methods

3.1 Animal Specimens

3.1.1 Ethical Approval

Ethical approval for the testing of animal organs for hantaviruses was obtained from the Faculty of Health Sciences' Committee for Experimental Animal Research (CEAR). Project number P09/02/001 was assigned to the study.

Ethical approval for trapping by Dr Sonja Matthee (Department of Conservation Ecology and Entomology) was obtained from the Faculty of Science Ethical committee (2006B01007).

3.1.2 Animal Trapping

Permits for the trapping of small mammals were obtained from the relevant authorities: Cape Nature (Permit No. AAA 004-00034-0035), and the Department of Tourism, Environment and Conservation of the Northern Cape Province (Permit No. 2268/2007).

Trapping was conducted by Dr Sonja Matthee and her research team for the study of macroparasites in various rodents in Southern Africa. The animals were also used for other non-virological research projects. Animals were trapped at selected sites in the Western Cape and Northern Cape Provinces representing different biomes and vegetation types: Fynbos, Succulent Karoo, desert, Dwarf shrub and mixed tree shrub (Matthee *et al* 2009).

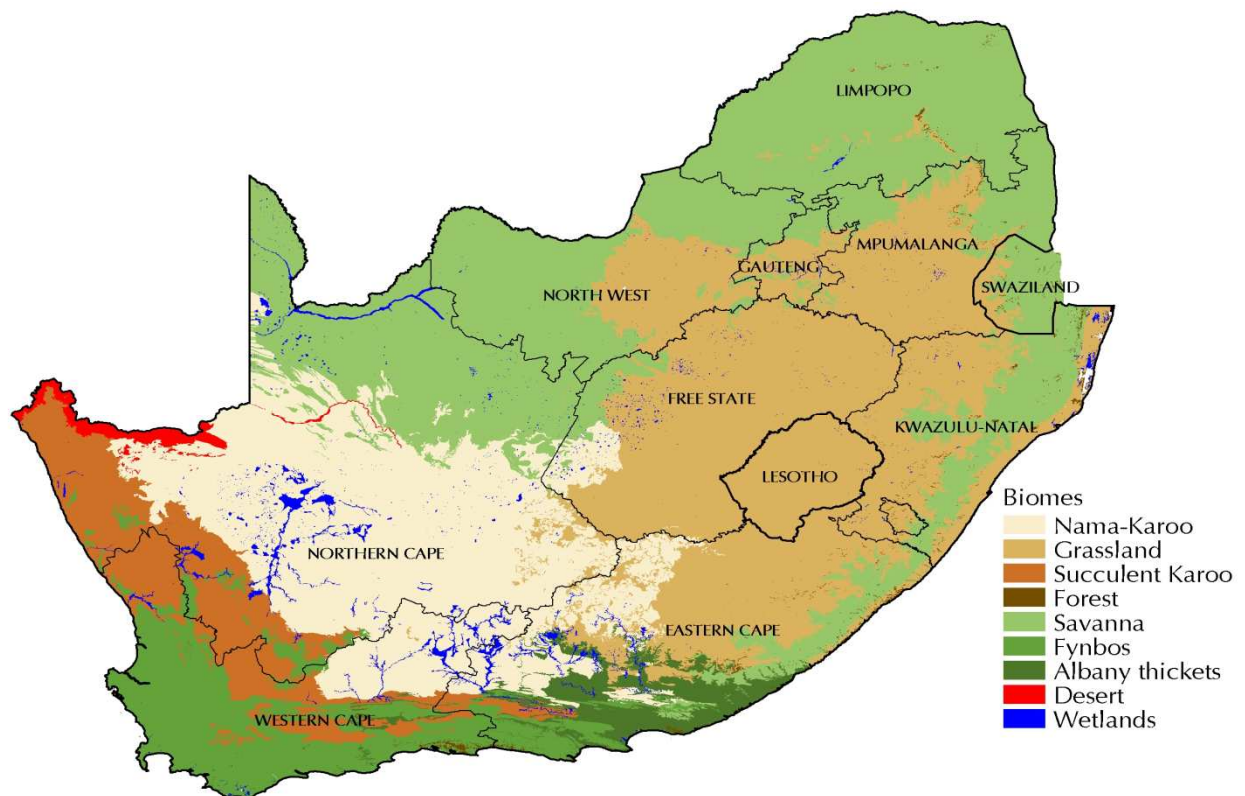


Figure 3.1 South African biomes.

A map of South Africa illustrating the different biomes within the country. Trapping was conducted in the Western Cape and the Northern Cape provinces, which consist of desert, Fynbos, succulent karoo, nama-karoo and desert [Source: <http://www.environment.gov.za/enviro-info/nat/biome.htm>]

Animal trapping was also conducted close to and within rural and urban areas, and on farms close to human dwellings. Adult rodents and shrew were mainly targeted as previous studies have shown that the highest antibody prevalence is associated with older animals. 30 adult animals with a body mass of at least 40g were trapped per locality. Trapping was done during reproductively active season for rodents and shrews which is mainly during spring and summer. Studies have shown that fighting and biting during the breeding season facilitates the transmission and since male animals tend to be more aggressive towards competing males during the breeding season (Hinson *et al*, 2004; Douglass *et al*, 2007), this increases the chances of recording positive animals.

Sherman-type live-traps were used in a standardized grid system over a period of 7 to 14 days per locality; wherever possible the traps were placed in the shade under shrubs or trees to minimise exposure of the animals to direct sunlight preventing the

animals from suffering extreme heat or cold. The traps were baited with a peanut butter-oats mixture and checked twice daily (mornings and afternoons) to optimise trapping success (Matthee & Krasnov, 2009).

Non-targeted species were identified and released unharmed at the trapping site. Targeted species were immediately euthanized by intra-peritoneal injection with 200mg/kg of Sodium Pentobarbitone, placed in pre-labelled plastic bags and stored in a field freezer. Once back at the laboratory the animals were stored at -80°C until dissection. 576 animals were trapped at different localities in the Western Cape Province, Northern Cape and Namibia, from 2007 to 2010 (Addendum C).

Animals were dissected and organs removed by Dr Matthee's team and transported on ice to the Division of Medical Virology at Tygerberg Campus. Upon arrival the samples were catalogued (specimen number and trapping location was recorded) and stored at -80°C to await RNA extraction and testing.

3.1.3 Tissue Disruption and Homogenisation

After thawing, ~20mg of lung tissue was cut from stored lung and chopped into small pieces using No.10 scalpels, which can fit into 2ml tube, and 350µl of Qiagen RLT buffer, a highly denaturing buffer which contains guanidine-thiocyanate which inactivates RNase to maintain the integrity of RNA in the samples was added to the sample. The mixture was then drawn in and out of a 2ml syringe to homogenise it. The homogenised samples were then placed in QIAshredder columns from Qiagen (Germany) and centrifuged for 5 minutes at 14000 rpm to remove debris. Supernatant for each sample was then placed in 2.0ml tubes compatible with the QIAcube extraction machine for RNA extraction.

3.1.4 RNA Extraction

Total RNA was extracted automatically from the supernatant prepared for each sample during the tissue disruption and homogenisation process, using the RNeasy mini kit on the QIAcube platform (Qiagen, Hilden, Germany) using the manufacturer's standard protocol for total RNA extraction from animal cells and tissue (www.qiagen.com/qiacube/standard/protocolview.aspx?StandardProtocolID=794). The procedure is based on a technology that combines the selective binding properties of silica membranes with the speed of microspin technology. After the

tissue was lysed and homogenised in the presence of RLT buffer; Ethanol was then added in order to facilitate the binding of the RNA to the silica membranes in the columns. Contaminants were then washed from the washed away and the RNA was eluted in 50µl of RNase-free water.

3.1.5 Hantavirus Screening

3.1.5.1 Preparation of Positive Control

Virus was grown under BSL-3 conditions in Vero E6 cells in 25cm² flasks in MEM containing 10% foetal calf serum, L-glutamate, penicillin and streptomycin. The medium was then changed weekly and two-thirds of the cells were trypsinised at 3-week intervals and passaged into new flasks. Fifty percent uninfected cells were added to the infected cell culture to make a 1:1 mixture. The cultures were then analysed at 7 day intervals by immunofluorescence assay (IFA) using hantavirus-specific monoclonal antibodies 4C3 and 1C12 at 5µg/ml since hantavirus infection does not cause visible cytopathic effect in infected cells (Razanskiene *et al*, 2004). The cells were first fixed with acetone onto a glass slide, and monoclonal antibodies were added and then incubated for 30 minutes at 37°C. Fluorescein isothiocyanate (FITC) conjugated rabbit anti-mouse antibodies were added to the slide and incubated for 30 minutes at room temperature, washed with PBS and viewed under fluorescence microscope. Cell cultures positive for hantavirus antigens were centrifuged at and the cell culture supernatant was stored at -80°C and extracted at later stage (Nemirov *et al*, 1999).

RNA was extracted from cell culture supernatant manually using the RNeasy mini kit from Qiagen (Germany). The RNA was reverse transcribed using the M-MLV reverse transcriptase kit from Invitrogen (USA). Reaction volume was 20µl with 10µl RNA template, 4µl 5× First Strand buffer, 0.5µl 200U/µl Moloney murine leukemia virus (M-MLV) reverse transcriptase, 0.5µl random hexamer primer(Bioline, Germany), 0.1µl 0.1M dithiothreitol, 3µl 10mmol/L dNTP mix from Bioline (Germany) 0.5µl 40U/µl Ribolock RNase inhibitor()and 1.4µl nuclease-free water from Qiagen (Germany). The RNA was then incubated under the following conditions: 25°C for 10 minutes, 42°C for 10 minutes and 96°C for 6 minutes on the 9700 GeneAmp® PE cyclor from Applied Biosystems (California, USA).

3.1.5.2 cDNA Synthesis

Reverse transcription was performed using the M-MLV reverse transcriptase kit from Invitrogen (USA). The M-MLV RT uses single-stranded RNA as a template to synthesise cDNA in the presence of primer; Random Hexamer primers from Bioline (Germany) were used for reverse transcription, which was run on the GeneAmp 9700 PE from Applied Biosystems (California, USA) under the following conditions: 25°C for 10 minutes, 42°C for 10 minutes and 96°C for 6 minutes. Reaction volume was 20µl; 8µl RNA of each sample, 4µl 5x First strand buffer, random hexamer primers (Bioline), 0.5µl 200U/µl M-MLV RT, 0.1M DTT, dNTP mix (Bioline), 40U/µl Ribolock RNase inhibitor (Fermentas, UK) and 1.9µl nuclease-free water (Qiagen, Germany).

3.1.6 Pre-nested and Nested Amplification

Amplification was carried out using the primers from Klempa *et al*, 2006 (**Table 2.1**). The degenerate primers were designed to target a conserved region in the L-segment nucleotide sequence and to detect known and unknown members of the *Hantavirus* genus (Klempa *et al*, 2006). Pre-nested amplification was carried out using the forward primer HAN-L-F1 and reverse primer HAN-L-R1 producing a ~412bp fragment.

Reaction volume was 25µl with 5µl cDNA added to 12.5µl Thermo-Start™ PCR master mix from Thermo Scientific (UK) containing a chemically modified *Taq* DNA polymerase a hot-start enzyme which remains inactive at room temperature and requires activation at 95°C for 15 minutes, 0.2mM dNTPs and 1.5mM MgCl₂, 5µl forward primer, 5µl of reverse primer, 1µl MasterAmp™ 10x PCR enhancer from Epicentre® Biotechnologies (Wisconsin, USA) and 2.0µl of nuclease-free water (Qiagen, Germany) and amplification done on the 9700 PE Applied Biosystems with the following cycling conditions: 95°C for 15 minutes, 95°C for 30 seconds, 53°C for 45 seconds and 72°C for 40x cycles, and 72°C for 6 minutes.

For nested amplification the forward primer HAN-L-F2 and reverse primer HAN-L-R2 were used to produce a ~390bp fragment. The total reaction volume was 50µl; 1µl pre-nested product was added to 25µl Thermo-Start™ PCR master mix, 5µl forward primer, 5µl of reverse primer, 1µl MasterAmp™ 10x PCR enhancer and 13µl of nuclease-free water. Amplification reactions carried out at the following cycling

conditions: 95°C at 15 minutes, 95°C for 30 seconds , 53°C for 45 seconds, 72°C for 30 seconds for 25x cycles and 72°C for 6 minutes.

Reverse transcription, pre-nested and nested amplification were carried out in 0.2ml 8 strip, thin-walled PCR tubes from Axygen (USA).

Table 3.1 PCR primers for the pre-nested and nested amplification

The primers target the conserved region of the L-segment.

Primers	Sequences (5'→3')
HAN-L-F1	ATG TAY GTB AGT GCW GAT GC
HAN-L-R1	AAC CAD TCW GTY CCR TCA TC
HAN-L-F2	TGC WGA TGC HAC IAA RTG GTC
HAN-L-R2	GCR TCR TCW GAR TGR TGD GCA A

3.1.7 Gel Electrophoresis

Nested PCR products were visualized on 1% (m/v) agarose gel. Five hundred millilitres 0.05M EDTA solution, pH 8.0 was prepared from 146.1g EDTA (Sigma Aldrich, USA) and distilled water,. 10M NaOH (Merck, Germany) was used to adjust the pH and the solution was then autoclaved at 121°C for 1 hour. 50x TAE buffer was prepared using 57.1 ml of glacial acetic acid (Merck Chemicals, Germany), 242g Tris base (Boehringer Mannheim, USA), 100ml of 0.05M EDTA and distilled water. The 1% agarose was prepared by mixing 1g of LE agarose (Promega, USA) and 99ml of 1x TAE buffer; the mixture was microwaved on high for 3 minutes and then poured into an electrophoresis tray and allowed to set for at least 30 minutes. The electrophoresis buffer (1x TAE) was added to the tray to submerge the gel. Samples were loaded using 6x orange/blue loading dye (Promega, USA) and a 100bp ladder (Bioline, Germany) was used as the molecular marker. Five microlitres of 0.05 mg/μl ethidium bromide (Promega, USA) was added to the electrophoresis buffer (1xTAE) and a power supply of 60V was used for electrophoresis for approximately 45 minutes.

3.1.8 Visualisation of PCR Product

Nested PCR product was visualised on the UVItec Alliance (Chemiluminescence and Fluorescence system) (Cambridge, UK) using the UVIchemi image acquisition system to capture the gel images by exposing the gels to UV light at 254nm to visualise the PCR products, the expected band size is ~390bp. The images were then enhanced and edited using UVIband software, and saved.

3.2 Screening of Human Specimens

3.2.1 Ethics Approval

Ethical approval for recruitment of patients from Tygerberg hospital and Groote Schuur hospital was obtained from the Stellenbosch University Human Research Ethics Committee (HREC) (Ethics Reference No: N09/02/058) (Addendum D).

3.2.2 Sampling

3.2.2.1 Patients with Acute, Suspected Hantavirus-related Disease

A surveillance system for patients expressing the symptoms associated with possible hantavirus infection was established with the help of infectious disease, renal and pulmonology physicians at the participating hospitals. Patients who fulfilled a clinical definition of possible hantavirus disease were recruited to the study: fever of unknown origin, i.e. not responding to antibiotic treatment, plus any of the following also with unknown cause: acute renal failure, acute respiratory failure, acute liver failure, or mucosal bleeding. Patient data such as date of birth, place of residence, symptoms and location of potential exposure to rodents are recorded and 10ml of blood collected in yellow- top Silica Clot Activator vacutainer tubes (BD Biosciences).

3.2.2.2 Opportunistic (convenience) Serosurvey

This survey is conducted using residual patient serum samples sent to NHLS laboratories in the Western Cape for routine laboratory testing that are leftover after the requested tests are completed. The samples being used would otherwise have been discarded. Since the purpose of screening these samples was determine whether Hantavirus antibodies are present in the population, samples were anonymised upon receipt and no information on demographics or medical

information on the patient was recorded. However, source laboratory, age and sex were recorded.

3.2.3 In-house Indirect ELISA

An indirect ELISA was set up according to published protocols (Dargeviciute *et al*, 2002; Razanskiene *et al*, 2004) to detect hantavirus-specific IgG antibodies using recombinant N protein from Puumala and Dobrava viruses. Neither of these viruses has been isolated in Africa but these antigens are available and it can be expected that there will be some degree of antibody cross-reactivity where antibodies against a specific virus are able to bind to corresponding antigens of viruses closely related to the one that induced the antibody production (Elgh *et al*, 1998).

The N protein has a molecular weight of about 50kDa and contains 429 to 433 amino acid residues (Kaukinen *et al*, 2005). It is the most abundant hantavirus protein synthesized during the early steps of infection. A primary function of the N proteins is to protect the RNA genome from nuclease degradation. The N protein in the ribonucleoprotein (RNP) interacts with the cytoplasmic tail of the Gc to initiate virion assembly (St Jeor *et al*, 2005). Studies in humans and animal models have shown that the N protein is highly immunogenic (Dargeviciute *et al*, 2002).

3.2.4 Expression of Recombinant N Protein

The expression and preparation of the recombinant nucleoproteins for the Dobrava and Puumala viruses was done at the Institut für Med. Virologie, Chaité Universitätsmedizin, Berlin, Germany.

The open reading frames for the N protein for the Puumala and the Dobrava viruses were cloned separately into the pFX7, a yeast expression vector. pFX7 contains formaldehyde resistance gene and expression of the N protein is under the control of the hybrid GAL10-PYK1 promoter (Dargeviciute *et al*, 2002). The plasmids were then transformed into *Saccharomyces cerevisiae* cells, strain FH4C and the yeast cells were incubated on a YEPD agar plate (1% yeast extract (Difco, Germany), 2% peptone, 2% glucose (Merck, Germany) and 5mM of formaldehyde (Carl Roth GmbH, Germany) for 24 hours at 30°C.

Most of the colonies from the plate were then inoculated into fresh YEPD medium and incubated for 24 hours at 30°C on a shaker. The induction medium, 75ml of

YEPD medium supplemented with 3% galactose (Carl Roth GmbH, Germany) and 75µl formaldehyde was added to the yeast cells and the cells were then grown on a shaker for another 18 hours at 30°C.

Yeast cells were harvested and washed with 10ml of 20mM pH 7.6 PBS (Biochrom, Germany) and centrifuged at 2000g for 20 minutes at 4°C. The supernatant was then discarded; the cell pellet was weighed and re-suspended in disruption buffer (50mL of 20mM, pH 7.6 PBS, 29g of 2mM EDTA (ICN Biomedicals GmbH, Germany) and 500µl 1mM PMSF (Boehringer/Roche, Germany)). An equal volume of glass beads (Sigma, USA) was added and the cells were disrupted by vortexing for 10 minutes. The yeast lysate was then centrifuged at 10 000g for 40 minutes, supernatant discarded and the cell pellet was re-suspended in 10ml of extraction buffer (50mL of 20mM, pH 7.6 PBS, 500µl of 1mM PMSF (Boehringer/Roche, Germany) and 1% Tween (Serva Electrophoresis, Germany)), shaken at 4°C for 30 minutes and centrifuged at 10 000g for 30 minutes in order to remove the contaminating proteins (Dargevicuite *et al*, 2002). The N protein-containing pellet was then re-suspended in 10ml of re-suspension buffer (6M Guanidine-hydrochloride (ICN Biomedicals GmbH, Germany), 0.1M NaH₂PO₄ (Roth, Germany) and 0.01M Tris-Cl (Roth, Germany), pH 8) and shaken at 200rpm and room temperature for 1 hour, and centrifuged for 20 minutes at 10 000g and room temperature. Two millilitres of Ni²⁺-NTA agarose (Qiagen, Germany) was added to the re-suspended cells, the mixture was then added to a polypropylene column (Qiagen, Germany) and the column was washed with Qiagen buffers Buffer B (10ml), buffer C (20ml) and the protein washed with buffer D. The protein was then eluted 3x with 2ml of buffer E and collected in 2ml fractions. The purity of recombinant Puumala (PUU-rN) and Dobrava (DOB-rN) nucleocapsid proteins was determined on a 15% SDS-PAGE gel stained with Coomassie-blue. The specificity of the recombinant N protein was tested in Western blot assay using monoclonal antibodies: Puumala-specific (A1C5) and cross-reactive for Hantaan, Dobrava and Puumala virus (1C12 or E5G6). The bands observed on the Western Blot are for the PUU-rN-Vranica and DOB-rN-Slovakia with respective molecular weights of 50.3 kDa and 49.1 kDa. The purified N proteins were aliquoted and stored at 4°C until further use.

Before use in the assay antigens PUU-rN and DOB-rN were diluted in the coating buffer with a final concentration of 0.3µg/ml: the PUU-rN stock had a concentration of 445µg/ml, 10µl of stock was added to 14823.3 µl coating buffer. The concentration of DOB-rN stock was 340µg/ml; 10µl of stock was mixed with 11323.3µl coating buffer. Working concentrations of both antigens were stored at 4°C.

3.2.5 Preparation of ELISA Buffers

Coating buffer was prepared by diluting 3.03g of Na₂CO₃ (Sigma Aldrich, Germany) and 6.0g of NaHCO₃ (Sigma Aldrich, Germany) in water to make a 0.05 M 1L solution with pH 9.6. The pH of the solution was confirmed with the pH strips (Sigma Aldrich, USA). The Coating buffer can be stored for 2 weeks at room temperature.

One gram of bovine serum albumin (BSA) fraction V (Sigma Aldrich, Germany) was diluted in Dulbecco's phosphate buffered saline (DPBS) without Ca²⁺ and Mg²⁺ (Biowhitaker, USA) to make 100ml 1% (m/v) BSA solution, which was stored at 4°C.

The 3% (m/v) BSA Blocking buffer was prepared with 3g of BSA fraction V added to DPBS to make a 100 ml solution which was also stored at 4°C.

For preparation of 10× wash buffer: a 1.0 M Tris-Cl was prepared by adding 12.11g of Tris ultrapure grade (Sigma Aldrich, Germany) to 80ml of distilled water. Seven millilitres of HCl (Sigma Aldrich, Germany) was added and then more water was added to make up a 100ml solution. The solution was then autoclaved at 121°C for 1 hour. Ten millilitres of 1.0 M Tris-Cl, 17.56g of NaCl (Sigma Aldrich, Germany) and 1ml of Tween[®] 20 (Sigma Aldrich, Israel) were added together and distilled water was added to make a 100ml solution. To make 1× wash buffer (working solution) 50ml of 10× wash buffer was mixed with 450ml of water and the solution was stored at room temperature.

Stop solution, 1.0 M H₂SO₄ was prepared by diluting 18M H₂SO₄ (Sigma Aldrich, Germany) in distilled water; 5ml of 18.0 M H₂SO₄ was slowly added to 85ml of water. The solution was stored at room temperature.

3.2.6 Positive and Negative Controls for ELISA

The positive controls were obtained from the National Reference Laboratory for Hantaviruses at the Institut für Med. Virologie at Charité Universitätsmedizin in Berlin, Germany. Two IgG antibody positive control sera were used: H5 obtained from a patient infected with Dobrava (Slovakia) virus, H7 from a patient infected with Puumala virus. Both samples tested positive on the in-house IgG ELISA and results were confirmed by Western blot and Focus Reduction Neutralisation test (FRNT).

Negative control sera were obtained from healthy donors with no history of an illness compatible with hantavirus disease in the Division of Medical Virology, Tygerberg, South Africa. Samples were tested for hantavirus-specific antibodies in duplicate using the ELISA assay; all the samples tested were negative and were subsequently used as negative controls in the assay.

3.2.7 Screening of Patient Sera by ELISA

Nunc® Polysorp 96-well microtitre plates (Nalgene, Denmark) were used. Two wells were allocated to each sample as well as the positive and negative controls. The first test well was coated with 100µl of antigen and the second control well was mock-coated with the 100µl sample dilution buffer. Each plate was prepared according the template below (figure 3.2); the plate was divided in half, the first 6 columns were allocated for testing reactivity to DOB-rN and column 7 to 12 were allocated to PUU-rN.

1	2	3	4	5	6	7	8	9	10	11	12
Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test
POS DOB	POS DOB	SAMPLE 8	SAMPLE 8	SAMPLE 16	SAMPLE 16	POS PUU	POS PUU	SAMPLE 8	SAMPLE 8	SAMPLE 16	SAMPLE 16
SAMPLE 1	SAMPLE 1	SAMPLE 9	SAMPLE 9	SAMPLE 17	SAMPLE 17	SAMPLE 1	SAMPLE 1	SAMPLE 9	SAMPLE 9	SAMPLE 17	SAMPLE 17
SAMPLE 2	SAMPLE 2	SAMPLE 10	SAMPLE 10	SAMPLE 18	SAMPLE 18	SAMPLE 2	SAMPLE 2	SAMPLE 10	SAMPLE 10	SAMPLE 18	SAMPLE 18
SAMPLE 3	SAMPLE 3	SAMPLE 11	SAMPLE 11	SAMPLE 19	SAMPLE 19	SAMPLE 3	SAMPLE 3	SAMPLE 11	SAMPLE 11	SAMPLE 19	SAMPLE 19
SAMPLE 4	SAMPLE 4	SAMPLE 12	SAMPLE 12	SAMPLE 20	SAMPLE 20	SAMPLE 4	SAMPLE 4	SAMPLE 12	SAMPLE 12	SAMPLE 20	SAMPLE 20
SAMPLE 5	SAMPLE 5	SAMPLE 13	SAMPLE 13	SAMPLE 21	SAMPLE 21	SAMPLE 5	SAMPLE 5	SAMPLE 13	SAMPLE 13	SAMPLE 21	SAMPLE 21
SAMPLE 6	SAMPLE 6	SAMPLE 14	SAMPLE 14	SAMPLE 22	SAMPLE 22	SAMPLE 6	SAMPLE 6	SAMPLE 14	SAMPLE 14	SAMPLE 22	SAMPLE 22
SAMPLE 7	SAMPLE 7	SAMPLE 15	SAMPLE 15	SAMPLE 23	SAMPLE 23	SAMPLE 7	SAMPLE 7	SAMPLE 15	SAMPLE 15	SAMPLE 23	SAMPLE 23
Wells coated with DOB-rN						Wells coated with PUU-rN					
Control wells are coated with sample dilution buffer											
Test wells are coated with recombinant N antigen (rN)											

Figure 3.2 Template for the ELISA plates.

The plates were then incubated for 1 hour at 37°C in the Nuaire NU-4850-E to facilitate binding of the antigen to the wells (NuAire Inc., USA). Thereafter the contents of the wells were discarded and the plate gently tapped against a paper towel to remove the remaining fluid. 150µl of the blocking buffer was then added to the wells in order to block any spaces in the wells where no antigen bonded, thus decreasing the occurrence of non-specific binding of antibodies in sera, and the plates were incubated at room temperature for 1 hour. Following incubation the blocking buffer was discarded in the same way as the coating and dilution buffer. The plates were then washed 4x with wash buffer using the MultiWash II plate washer (Tricontinent, USA) to remove unbound blocking buffer and antigens.

The patient sera and the controls were then diluted (1:200) in sample dilution buffer and 100µl of sera and negative control was added to the respective wells. The plates were then incubated for 1 hour at 37°C to facilitate binding of any antibodies present in the sera to the antigen. After incubation the contents of the wells were discarded and 4x wash was done to remove any antibodies that did not bind to the antigen.

The goat anti-human IgG HRP-conjugated antibody (Promega, USA,) specific for both heavy and light chains on human antibodies was diluted in the sample dilution buffer with a final concentration of 0.4µg/ml and 100µl of the diluted antibody was added to all the wells whereupon the plate was incubated again for 1 hour at 37°C. The HRP-conjugated antibody was added to bind to the human antibodies present.

The contents of the wells were discarded and the 4x wash repeated, then 100µl of BD Opt[®] EIA TMB substrate was prepared according to the instructions given by the manufacturer (BD Biosciences, USA) was added to the wells and then the plate was incubated for 20 minutes at room temperature in the dark. TMB substrate is a chromogenic substrate that produces a coloured product in the presence of HRP, resulting in the formation of blue colour in the positive wells. Then 100µl of stop solution a 1.0 M Sulphuric acid (Sigma Aldrich, Germany) was added to each well and colour change was observed in the reactive wells from blue to yellow.

The plate was read on the ELISA plate reader (Worldwide Diagnostics, USA) at 450nm to measure optical density in each well, using air as the blank.

For each test well, the optical density (OD) value for the control well was subtracted from the OD value for the test well to obtain the net OD of each sample to DOB-rN and PUU-rN: **Test OD₄₅₀ – Control OD₄₅₀ = antibody activity (net OD₄₅₀)**

Samples were considered positive if the value obtained was greater than or equal to 0.150 (Klempa *et al*, 2010).

CHAPTER 4

4 Results

4.1 Animal Trapping

A total of 576 rodent specimens were obtained for screening. Most of the animals were trapped at localities in the Western Cape, some in the Northern Cape and only one animal was trapped in Southern Namibia (Addendum C). The majority of the animals trapped were *Rhabdomys pumilio*. Table 4.1 below shows the distribution of the species that were trapped.

Table 4.1 Rodents and shrews trapped for the study, 2007-2009.

Species	Number of specimens
<i>Rhabdomys pumilio</i>	513
<i>Gerbilluris paeba</i>	9
<i>Rattus rattus</i>	13
<i>Otomys unisulcatus</i>	6
<i>Aethomys sp.</i>	6
<i>Otomys irroratus</i>	2
<i>Saccostomys sp</i>	1
<i>Parotomys sp</i>	1
<i>Mus musculus</i>	6
Shrews: <i>Soricidae</i> family (species not determined, classification based on physical features)	6
TOTAL	576

4.2 Hantavirus Screening

The reverse-transcribed Dobrava virus cDNA was amplified and visualised on a 1% agarose gel. Ten-fold serial dilutions of the cDNA were prepared down to 100 000x dilution, amplified and visualised on a 1% gel.



Figure 4.1 Pre-nested amplification product of the positive control.

1% agarose gel picture of the pre-nested amplification product of the cDNA positive control. After the first round of amplification, the 1000x and 100x are clearly amplified. Faint bands of the 10 000x are visible as well. Size of the bands is ~412bp

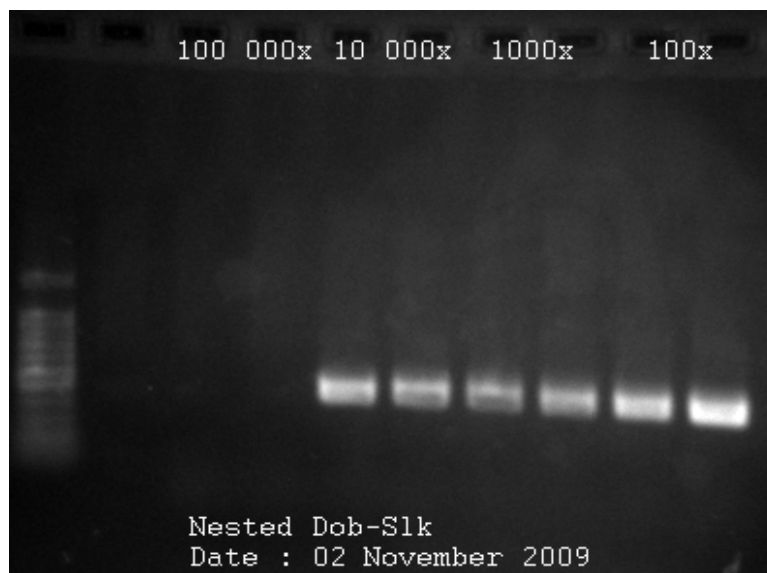


Figure 4.2 Nested amplification product of the positive control

1% agarose gel picture of the nested amplification product of the cDNA positive control. After nested amplification the product of 10 000x is visualised on the gel. Size of band is ~390bp

When screening specimens for hantavirus RNA, the positive control was used at 10 000x dilution. The objective was to use the lowest concentration of positive control at which strong amplification bands can be observed, to be economical with the control material without compromising the quality of the assay run, by ensuring that it has been efficient.

RNA was extracted from the lungs of all 576 animals that were trapped. All the samples were screened by pre-nested and nested Pan-Hanta PCR. All assay runs were valid, with positive and negative controls showing the expected results. All specimens tested negative when the nested PCR product was visualised on 1% agarose gel. Several samples tested positive on the first run; to confirm these result, RNA was extracted again from the remaining tissue and amplified again by PCR. On repeat testing, all these samples tested negative, suggesting that the positive result observed initially was due to cross-contamination from the positive control well since the samples were amplified in 0.2ml 8-well PCR strips (Axygen, USA).

4.3 Screening of Patient Sera by ELISA

4.3.1 Patients with Acute, Suspected Hantavirus-related Disease

No samples were received from patients with acute illnesses that might possibly hantavirus diseases were collected from hospital wards.

4.3.2 Opportunistic (convenience) Serosurvey

One hundred and sixty one residual sera samples were collected from the National health laboratory services (NHLS) at Tygerberg hospital tested for the presence of hantavirus specific antibodies; 99 of the specimens were from female patients and 62 from male patients (Addendum D). All samples were anonymised, with only age and sex recorded from the hospital stickers on the blood tubes.

Fourteen of the samples were obtained from males under the age of 30, 19 were from patients between age 30 and 50 years and 27 of the sera originated from patients over the ages of 50. Twenty-three of the specimens originated from female patients under 30 years of age, 37 from patients between age 30 and 50 years, and 38 from female patients over the age of 50. Age could not be recorded for 1 specimen from a male and from 2 female patients.

Table 4.2 Optical density OD₄₅₀ values for the positive control sera.

Dobrava (H5)	0.703
Puumala (H7)	0.541

Table 4.3 Results DOB-rN ELISA.

Net optical density at 450nm for each sample is stated. The samples with OD₄₅₀ ≥ to 0.150 were considered positive. Positive results are highlighted in bold.

Antibody activity - Net OD₄₅₀ DOB-rN

Specimen number		Specimen number		Specimen number	
HAN – 1	0.034	HAN - 55	0.174	HAN - 109	0.072
HAN – 2	0.094	HAN - 56	0.031	HAN - 110	0.053
HAN – 3	0.007	HAN - 57	0.181	HAN - 111	0.061
HAN – 4	0.045	HAN - 58	0.032	HAN - 112	0.045
HAN – 5	-0.011	HAN - 59	0.057	HAN - 113	0.030
HAN – 6	0.043	HAN - 60	0.074	HAN - 114	0.074
HAN – 7	0.025	HAN - 61	0.023	HAN - 115	0.067
HAN – 8	0.027	HAN - 62	0.132	HAN - 116	0.032
HAN – 9	-0.002	HAN - 63	0.398	HAN - 117	0.085
HAN – 10	0.042	HAN - 64	0.047	HAN - 118	0.040
HAN – 11	0.041	HAN - 65	0.123	HAN - 119	0.033
HAN – 12	0.029	HAN - 66	0.250	HAN - 120	0.047
HAN - 13	-0.004	HAN - 67	0.060	HAN - 121	0.191
HAN - 14	0.022	HAN - 68	0.118	HAN - 122	0.038
HAN - 15	0.067	HAN - 69	0.069	HAN - 123	0.066
HAN - 16	0.000	HAN - 70	0.016	HAN - 124	0.103
HAN - 17	0.016	HAN - 71	0.099	HAN - 125	0.165
HAN - 18	-0.001	HAN - 72	0.016	HAN - 126	0.074
HAN - 19	-0.002	HAN - 73	0.014	HAN - 127	0.039
HAN - 20	-0.002	HAN - 74	0.025	HAN - 128	0.036
HAN - 21	0.013	HAN - 75	0.023	HAN - 129	0.041
HAN - 22	0.017	HAN - 76	0.019	HAN - 130	0.056
HAN - 23	0.009	HAN - 77	0.036	HAN - 131	-0.019
HAN - 24	0.042	HAN - 78	0.023	HAN - 132	-0.024
HAN - 25	0.082	HAN - 79	0.026	HAN - 133	-0.031
HAN - 26	0.024	HAN - 80	0.036	HAN - 134	-0.010
HAN - 27	0.058	HAN - 81	0.022	HAN - 135	0.002
HAN - 28	0.013	HAN - 82	0.006	HAN - 136	-0.003

HAN - 29	-0.004	HAN - 83	0.032	HAN - 137	-0.073
HAN - 30	-0.013	HAN - 84	0.078	HAN - 138	-0.012
HAN - 31	0.192	HAN - 85	0.049	HAN - 139	-0.062
HAN - 32	0.094	HAN - 86	0.214	HAN - 140	0.097
HAN - 33	0.046	HAN - 87	0.064	HAN - 141	0.054
HAN - 34	0.674	HAN - 88	0.085	HAN - 142	0.081
HAN - 35	0.015	HAN - 89	0.024	HAN - 143	0.231
HAN - 36	0.031	HAN - 90	0.011	HAN - 144	0.102
HAN - 37	0.100	HAN - 91	0.168	HAN - 145	0.050
HAN - 38	0.042	HAN - 92	0.079	HAN - 146	0.026
HAN - 39	0.039	HAN - 93	0.019	HAN - 147	0.158
HAN - 40	0.030	HAN - 94	0.029	HAN - 148	0.077
HAN - 41	0.019	HAN - 95	0.016	HAN - 149	0.153
HAN - 42	0.019	HAN - 96	0.049	HAN - 150	0.046
HAN - 43	0.030	HAN - 97	0.147	HAN - 151	0.285
HAN - 44	0.068	HAN - 98	0.068	HAN - 152	0.046
HAN - 45	0.077	HAN - 99	0.017	HAN - 153	0.139
HAN - 46	0.143	HAN - 100	0.007	HAN - 154	0.009
HAN - 47	0.033	HAN - 101	0.266	HAN - 155	0.544
HAN - 48	0.047	HAN - 102	0.047	HAN - 156	0.061
HAN - 49	0.279	HAN - 103	0.016	HAN - 157	0.271
HAN - 50	0.063	HAN - 104	0.033	HAN - 158	0.062
HAN - 51	0.076	HAN - 105	0.043	HAN - 159	0.049
HAN - 52	0.063	HAN - 106	0.090	HAN - 160	0.104
HAN - 53	0.135	HAN - 107	0.190	HAN - 161	0.047
HAN - 54	0.215	HAN - 108	-0.005		

Table 4.4 Results PUU-rN ELISA.

Net optical density at 450nm for each sample is stated. The cut-off for positive values is ≥ 0.150 and positive results are highlighted in bold

Antibody activity - Net OD ₄₅₀ PUU-rN					
Specimen number		Specimen number		Specimen number	
HAN – 1	-0.036	HAN – 55	0.007	HAN – 109	0.069
HAN – 2	0.013	HAN – 56	0.059	HAN – 110	0.074
HAN – 3	0.028	HAN – 57	0.014	HAN – 111	0.093
HAN – 4	0.005	HAN – 58	0.006	HAN – 112	0.036
HAN – 5	0.002	HAN – 59	0.025	HAN – 113	0.115
HAN – 6	-0.021	HAN – 60	0.005	HAN – 114	0.067
HAN – 7	-0.031	HAN – 61	-0.007	HAN – 115	0.162
HAN – 8	0.011	HAN – 62	0.023	HAN – 116	0.029
HAN – 9	0.004	HAN – 63	0.050	HAN – 117	0.036
HAN – 10	0.001	HAN – 64	-0.008	HAN – 118	0.021
HAN – 11	0.023	HAN – 65	0.035	HAN – 119	0.084
HAN – 12	-0.015	HAN – 66	0.044	HAN – 120	0.064
HAN – 13	0.022	HAN – 67	0.001	HAN – 121	0.132
HAN – 14	0.006	HAN – 68	0.005	HAN – 122	0.036
HAN – 15	0.062	HAN – 69	0.012	HAN – 123	0.038
HAN – 16	0.003	HAN – 70	0.013	HAN – 124	0.077
HAN – 17	-0.018	HAN – 71	0.025	HAN – 125	0.129
HAN – 18	-0.009	HAN – 72	0.162	HAN – 126	0.027
HAN – 19	-0.005	HAN – 73	0.005	HAN – 127	0.046
HAN – 20	0.007	HAN – 74	0.044	HAN – 128	0.034
HAN – 21	0.001	HAN – 75	0.056	HAN – 129	0.051
HAN – 22	0.031	HAN – 76	0.061	HAN – 130	0.046
HAN – 23	-0.009	HAN – 77	0.065	HAN – 131	0.072
HAN – 24	-0.003	HAN – 78	0.032	HAN – 132	0.072
HAN – 25	-0.003	HAN – 79	0.026	HAN – 133	0.047
HAN – 26	0.015	HAN – 80	0.043	HAN – 134	0.089
HAN – 27	0.015	HAN – 81	0.009	HAN – 135	0.038
HAN – 28	0.044	HAN – 82	0.021	HAN – 136	0.048
HAN – 29	0.014	HAN – 83	0.029	HAN – 137	0.067
HAN – 30	0.018	HAN – 84	0.504	HAN – 138	0.055
HAN – 31	0.014	HAN – 85	0.025	HAN – 139	0.032
HAN – 32	0.017	HAN – 86	0.092	HAN – 140	0.100
HAN – 33	0.015	HAN – 87	0.103	HAN – 141	0.039

HAN – 34	0.006	HAN – 88	0.166	HAN – 142	0.081
HAN – 35	0.033	HAN – 89	0.033	HAN – 143	0.106
HAN – 36	0.013	HAN – 90	0.021	HAN – 144	0.072
HAN – 37	0.038	HAN – 91	0.078	HAN – 145	0.031
HAN – 38	0.004	HAN – 92	0.101	HAN – 146	0.025
HAN – 39	0.013	HAN – 93	0.032	HAN – 147	0.154
HAN – 40	0.018	HAN – 94	0.072	HAN – 148	0.05
HAN – 41	0.107	HAN – 95	0.055	HAN – 149	0.073
HAN – 42	0.008	HAN – 96	0.031	HAN – 150	0.049
HAN – 43	0.010	HAN – 97	0.058	HAN – 151	0.247
HAN – 44	0.024	HAN – 98	0.114	HAN – 152	0.072
HAN – 45	0.005	HAN – 99	0.046	HAN – 153	0.115
HAN – 46	0.011	HAN – 100	0.025	HAN – 154	0.048
HAN – 47	0.005	HAN – 101	0.099	HAN – 155	0.041
HAN – 48	0.002	HAN – 102	0.044	HAN – 156	0.057
HAN – 49	0.011	HAN – 103	0.044	HAN – 157	0.203
HAN – 50	0.003	HAN – 104	0.045	HAN – 158	0.056
HAN – 51	0.006	HAN – 105	0.068	HAN – 159	0.052
HAN – 52	0.011	HAN – 106	0.090	HAN – 160	0.048
HAN – 53	0.003	HAN – 107	0.152	HAN – 161	0.043
HAN – 54	0.006	HAN – 108	0.146		

Table 4.5 Samples positive for DOB IgG antibodies, with details of the patient.

Sera with antibodies reactive to DOB-rN			
Specimen number	Net OD₄₅₀ DOB-rN	Age	Sex
HAN – 31	0.192	33	M
HAN – 34	0.674	51	M
HAN – 49	0.279	76	F
HAN – 54	0.215	48	F
HAN – 55	0.174	30	F
HAN – 63	0.398	39	F
HAN – 66	0.250	52	M
HAN – 86	0.214	65	M
HAN – 91	0.168	50	M
HAN – 101	0.266	45	M
HAN – 107	0.190	34	M
HAN – 121	0.191	38	F
HAN – 143	0.231	59	M
HAN – 147	0.158	37	F
HAN – 149	0.153	49	M
HAN – 151	0.285	68	M
HAN – 155	0.544	13	M
HAN – 157	0.271	49	F

Table 4.6 Samples positive for PUU IgG antibodies, with details of the patient.

Sera with antibodies reactive to PUU-rN			
Specimen number	Net OD₄₅₀ PUU-rN	Age	Sex
HAN – 72	0.162	27	F
HAN – 84	0.504	54	F
HAN – 88	0.166	45	F
HAN – 107	0.152	34	M
HAN – 115	0.162	28	F
HAN – 147	0.154	37	F
HAN – 151	0.247	68	M
HAN – 157	0.203	49	F

Table 4.7 Percentages of reactive specimens for each antigen and for both antigens

Antibody activity	Females	Males	
DOB-rN	7	11	18/161 (11.18%)
PUU-rN	6	2	8/161 (4.97%)
PUU-rN and DOB-rN	2	2	4/161 (2.48%)
Total	13	13	26

CHAPTER 5

5 Discussion

5.1 History of Hantaviruses

After the identification of Hantaan virus, the prototype of the genus, and identification of its host *Apodemus agrarius coreae* in 1978 by Lee *et al*, the search for hantavirus and respective reservoir hosts intensified. Hantaan virus has since been detected in Korea, China, Monogolia, as well as eastern Russia. Seoul and related hantaviruses have been identified in most of Asia, the Americas, and Europe and in 13 African countries since its hosts, members of the *Rattus* genus, are found on every continent with the exception of Antarctica (Lee, 1996). In Europe, Puumala and Dobrava hantaviruses were identified during the 1980s and 1990s, respectively (Harper & Meyer, 1999).

The Thottapalayam virus (TPMV) was first isolated in India in 1964 from *Suncus murinus* (Asian House Shrew) and was initially classified as an arbovirus. However subsequent studies showed that TPMV has bunyavirus morphology and the conserved terminal panhandle nucleotide signature carried by all known hantaviruses (Maes *et al*, 2007). It was the first hantavirus isolated from a non-rodent host. Since then numerous shrew-borne hantaviruses have been identified in Asia, Europe, North and South America such as the Asama virus (*Urotrichus talpoides*), American Ash river virus and Jemez Springs virus in the Masked Shrew (*Sorex cinereus*) and Dusky Shrew (*Sorex monticolus*), Cao Bang virus in Chinese shrew mole (Song *et al*, 2007b) and Seewis virus in the Eurasian common shrew (*Sorex araneus*) (Song *et al*, 2007c).

The prospect hill virus which is non-pathogenic was the first American hantavirus to be identified in *Microtus pennylvanicus* and *Peromyscus leucopus* (Lee *et al*, 1982). The outbreak of hantavirus cardiopulmonary syndrome (HCPS) in the Four Corners region, USA led to the identification of its causative agent the Sin Nombre virus and its host *Peromyscus maniculatus* (Harper & Meyer, 1999). Various other North American viruses and hosts were identified in other parts of the USA and Canada as well. The first South American HCPS cases were reported in Brazil in 1994, and the

only cases of human-to-human transmission of hantavirus infection was observed during the Andes virus outbreak in Argentina in 1996 (Martinez *et al*, 2005).

There are approximately 150 000 cases of Haemorrhagic fever with renal syndrome (HFRS) reported worldwide every year, most of them in China and several thousand cases of Nephropathia epidemica (NE) are reported in Europe annually (Sjölander & Lundkvist, 1999) while approximately 2500 cases of hantavirus cardiopulmonary syndrome (HCPS) have been reported so far in the Americas (Vaheiri *et al*, 2008). Hantaviruses have rarely been investigated outside the regions where clinical disease is frequently observed despite their potential importance as human pathogens. A comparison of the geographic distribution of *Muridae* rodents and the associated hantavirus demonstrates that the currently known distribution of hantaviruses does not match the distribution of rodent hosts. Further rodent studies are therefore necessary, especially in Africa and South Asia in order to better understand the distribution of hantaviruses (Hettonen *et al*, 2008).

5.2 Evidence for Hantaviruses in Africa

The most recent serology study in Africa was a population-based survey carried out in Guinea, where the first African hantaviruses were identified. Seven hundred and seventeen samples were tested; 68 were from patients from Sangassou village and 649 were residual samples from a Lassa fever study performed in 2001. The patient sera were tested for the presence of IgG antibodies against Puumala, Dobrava and Sangassou viruses by ELISA using recombinant N proteins of the above mentioned viruses and results were confirmed by IFA and focus reduction neutralisation test (FRNT). ELISA was also employed to test for the presence of IgM antibodies. Results of the survey showed a 1.2% prevalence of hantavirus antibodies (Klempa *et al*, 2010).

No disease has been associated with SANGV and TGNV or any other African hantavirus, however the potential of these viruses and other unidentified hantaviruses to cause disease in humans cannot be ignored as it is possible that HFRS may be confused with other diseases such as other viral haemorrhagic fevers, rickettsiosis, plague, severe pneumonia and leptospirosis (Bi *et al*, 2008).

Previous surveys were done in the 1980s in Central and West African countries; Gonzalez *et al* conducted a survey in Cameroun, Central African Republic, Chad, Congo, Equatorial Guinea and Gabon; 2893 samples were collected randomly in 1985-87, and tested by IFA for the presence of hantavirus specific antibodies. The survey reported a prevalence of 6.15% (Gonzalez *et al*, 1989). A previous survey has been conducted in the Central African Republic between 1982 and 84 and reported 0.7% prevalence (Gonzalez *et al*, 1988). Studies were also conducted in Senegal (Saluzzo *et al*, 1985), Nigeria (Tomori *et al*, 1986) and Egypt (Bostros *et al*, 2004) has found evidence of hantavirus infection in humans. Two studies were conducted in Egypt the first conducted by Hoogstraal *et al* reported seroprevalence of 0-6% in adults and the Corwin *et al* study reported a prevalence of 9% in children (Corwin *et al*, 1992). Studies in Asia have found the hantavirus antibody prevalence varies from 0.48% in Japan and 33% in Thailand. In Europe seroprevalence is as low as 0-1.9% in Switzerland and as high as 24% in Puumala virus endemic areas in Norway. Prevalence in America is as high as 43% in the Chaco area in Panama where HCPS is endemic (Bi *et al*, 2008).

The first African hantavirus, Sangassou virus (SANGV), was identified in 2006; 1129 blood specimens from Guinea west Africa were screened using the degenerate pan-hanta primers targeting a conserved region on the L-segment of the viral genome (Klempa *et al*, 2006). Phylogenetic studies of the nucleotide and amino acid sequences of all three viral segments showed that the virus is most closely related to Dobrava virus (figure 5.1) (Klempa *et al*, 2006; Hettonen *et al*, 2008).

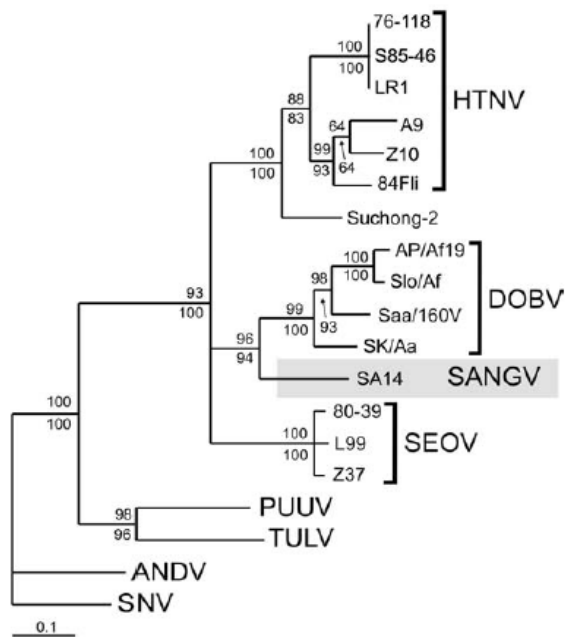


Figure 5.1 Maximum likelihood phylogenetic tree of some of the hantaviruses constructed using partial S segment sequences [Source: Klempa *et al*, 2006].

5.3 The Search for a Novel Southern African Hantavirus

In this study 576 animals were trapped, most of which are *Rhabdomys pumilio* which is the target species for the parasite study currently being conducted by Sonja Matthee. Trapping was focussed in limited localities; mostly in the Western Cape and some trapping was done in the Northern Cape and southern Namibia and only 6 different species were trapped in our study. In the Guinea study which resulted in the identification of the first two African hantaviruses, specimens from 17 different species were tested for the presence of hantaviruses. Another limitation is the lack of a positive control to be extracted and reverse-transcribed simultaneously with the specimens; a cDNA control was run with the reverse-transcribed RNA samples during the pre-nested and nested amplification.

Sero-epidemiologic survey was carried out by Lee *et al* (1999) in South Africa; rodent sera were collected from various rodent species trapped in the Northern Cape and Western Cape provinces. Hantavirus antibodies were detected in 2 of the 221 rodents, *Aethomys namaquensis* and *Tatera leucogaster* (Lee *et al*, 1999). In our study, only 6 *Aethomys* sp. were screened for the presence of hantavirus sequences and all tested negative.

Identification of a positive specimen would have resulted in subsequent sequencing and comparison of the obtained sequence to known members of the genus to determine, sequence similarity on a nucleotide and amino acid level by comparing S-, M- and L –segments to determine if a new species has been identified according to the criteria set out by the ICTV. Rodent or shrew trapping would then be conducted in the area from which the positive animal was trapped previously in order to determine prevalence of infection in the local population by RT-PCR. A recombinant antigen would be prepared of the new virus and used to set up an in-house serological assay, an ELISA.

An attempt would also have been made to isolate the novel virus from cultures, using Vero E6 cells. Successful virus isolation of the virus would allow, focus reduction neutralisation test (FRNT) based in the novel virus; it is the gold standard for serotyping hantaviruses. Sera from human beings near the trapping area would be tested to determine if the virus is the cause of human infections in specimens that test positive in ELISA and western Blot assays.

A sero-survey of the general population in the area or near the area the positive animal was tested would be conducted to determine the prevalence of hantavirus specific antibodies using the in-house with recombinant antigen based on the novel virus). This is the same approach that was followed Guinea following the identification of SANGV and TGNV; 68 serum samples were screened from residents with fever of unknown origin in Sangassou village retrospectively and IgG antibodies were detected in 3 of them. One of the positive subjects (S55) exhibited HFRS-like disease for 1 week and suffered from chills, headache, asthenia, myalgia, breathing difficulty, nausea, vomiting, oliguria, hypotonia, and oedema of the face and lower extremities (Klempa *et al*, 2010).

Serum taken from the S55 during the acute phase of illness tested positive for IgM antibodies (Table 5.1). The two other seropositive patients also had HFRS-like disease but it was less severe (Klempa *et al*, 2010). Below is a table showing the test results from patient S55.

Table 5.1 ELISA results from S55.

Post acute and post coalescent serum from the subject was tested [Source: Klempa *et al*, 2010].

Serum sampling	IgM ELISA results, OD		IgM titration		IgG ELISA results, OD		IgG titration	
	PUUV	SANGV	PUUV	SANGV	PUUV	SANGV	PUUV	SANGV
Postacute	0.402	2.246	1:400	1:1600	0.199	0.288	1:400	1:1600
Postconvalescent	—	—	—	—	0.355	0.428	1:400	1:800

NOTE. Minus signs indicate negative test results. Focus reduction neutralization test results are reported in Table 1. ELISA, enzyme-linked immunosorbent assay; IgG, immunoglobulin G; IgM, immunoglobulin M; OD, optical density; PUUV, Puumala virus; SANGV, Sangassou virus.

Though no novel hantavirus was isolated in this study, trapping will continue. Strategies will have to be put in place to improve the chances of identifying a Southern African hantavirus. Various research groups targeted rodents and shrews closely related to known reservoir hosts of hantaviruses. Arai *et al* identified a novel hantavirus in testing stored tissue from the northern short-tailed shrew (*Blarina brevicauda*) in the USA. The Imjin virus was identified in the Ussuri white-toothed shrew (*Crocidura lasiura*) closely related to TPMV host *Suncus murinus* (Song *et al*, 2009).

In southern Africa, 4 *Suncus* species occur and there are more than 103 *Crocidura* species, which occur in most of Africa with the exception certain parts of the Sahara. *Crocidura* species are the most common and most diverse shrew in Africa. They occur in all vegetation types and at all altitudes (Kingdon, 2003).

Another strategy would be target the species that tested positive in the seroepidemiologic study conducted in the late 1990s in South Africa (Lee *et al*, 1999), *Aethomys namaquensis* and *Tatera leucogaster* as well as to expand trapping into other ecosystems in South Africa, which should increase the variety of rodents and shrews being trapped. In the next phase of our study, more rodent trapping will be conducted in the coming spring and early summer. Plans are underway for trapping in Namibia to moving northwards into forest and woodland savannah as well.

5.4 Detection of Hantavirus-specific Antibodies in the South African Population

The diagnosis of HFRS and HCPS in human beings relies predominantly on serological testing. PCR has been used successfully in detecting PUU RNA in some patient specimens, but the short duration or absence of viraemia during the acute phase of infection means that other methods must be used for patient diagnosis (Sjölander & Lundkvist, 1999). Immunofluorescence assays (IFA) have been the traditional method of hantavirus serological diagnosis but it is less sensitive when compared to N protein-based ELISAs (Sjölander & Lundkvist, 1999; Meisel *et al*, 2006). The nucleocapsid protein is highly immunogenic in reservoir hosts, immunised laboratory animals as well as infected humans (Meisel *et al*, 2006). Viral antigens from hantavirus infected cells or recombinant N proteins are used in serological assays; recombinant N proteins (rN) are used in assays because N-specific antibodies occur early after infection and probably persist for life, the N protein can be expressed in *E.coli*, yeast and insect cells. The high level of conservation of the N protein among members of the genus means that cross-reaction between distantly related hantaviruses occurs; this allows for the detection of a majority of hantavirus infections by heterologous antigen but compromises a species-specific diagnosis should it be required, e.g. in areas where more than one hantavirus occurs and may cause diseases with different severity (Elgh *et al*, 1998; Sjölander & Lundkvist, 1999; Meisel *et al*, 2006).

In the in-house ELISA assay set up in our study, recombinant N antigens PUU-rN and DOB-rN prepared in yeast cells were used to detect anti-hantavirus IgG antibodies in patient sera collected in the Western Cape in a opportunistic serosurvey; 161 residual serum (clotted blood) samples from human patients sent for routine laboratory testing at the NHLS laboratories in Tygerberg Hospital that are left over after the requested tests have been done and would otherwise be discarded. Samples were tested against PUU-rN and DOB-rN and positive results were observed in 26 of the samples; 18 samples were reactive against DOB-rN, 8 were reactive against PUU-rN and 4 were reactive against both antigens.

Non-specific binding can result in false positives; it is therefore possible that some specimens especially those with OD₄₅₀ values equal to or close to 0.150 are false positives. Positive results on the on the ELISA assay will therefore be confirmed by Immunoblot assay and focus reduction neutralisation test (FRNT), although FRNT can only be performed once an indigenous virus is isolated from cell culture.

A sero-epidemiologic survey was conducted in South Africa in the late 1990s to determine the presence of hantavirus serologically. In this survey, sera were collected from patients with renal failure, pulmonary syndrome, and fever of unknown origin admitted to hospitals in Cape Town. Sera were also collected in the general population in the Eastern Cape and Western Cape provinces (Lee *et al*, 1999).

Sera were screened for hantavirus specific antibodies by immunofluorescence assay (IFA) using Hantaan, Seoul, Puumala, and Prospect Hill viruses. Sera positive in the IFA test then confirmed by a differential IFA test and plaque reduction neutralisation assay (PRNT). A prevalence of 0.57% was observed in the Eastern Cape and 2% prevalence was observed in the Western Cape. Of the sera from the recruited patients, 3 of the 318 patients diagnosed with chronic renal failure, acute respiratory distress syndrome and glomerulonephritis tested positive (Lee *et al*, 1999).

In the next phase of our study, the sera that tested positive on the IgG ELISA will be tested using more specific serological assays such as immunofluorescence assay (IFA) as well as the Western blot. This confirmatory testing is necessary as the screening ELISAs are known to produce a substantial rate of non-specifically reactive specimens (Chandy *et al*, 2009).

The recruitment of acutely ill patients with hantavirus-like disease fever of unknown origin, not responding to antibiotic treatment, along with any of the following of unknown aetiology: acute renal failure, acute respiratory failure, acute liver failure, pneumonia and/or mucosal bleeding will also commence. Information about signs and symptoms exhibited in the patients as well as possible location of exposure for the patients will be recorded (Addendum B).

Patients will be recruited at tertiary hospitals in Cape Town: Tygerberg hospital and Groote Schuur hospital with the assistance of physicians in the renal and respiratory care units at the respective hospitals. These specimens will be tested for IgG and IgM antibodies by ELISA using and any positive results will be confirmed by immunoblot assay and FRNT. Studying these patients will lead to the formation of a clinical case definition for hantavirus disease in South Africa.

Conclusion

In this study, an attempt was made to identify and isolate the first Southern African hantavirus. A total of 576 lung specimens from a variety of rodents and shrews trapped in the Western Cape, Northern Cape and southern Namibia were screened for the presence of hantavirus sequences using universal Pan-Hantavirus degenerate primers designed to detect all known and unknown hantaviruses. All specimens tested were negative for hantaviruses. However, the inability to identify a novel hantavirus does not mean that no hantaviruses are present in South Africa; the sample size is still relatively small, and it may have missed the geographical area and / or host species implicated. Of note, Lee *et al* (1999) reported on the presence of hantavirus-specific antibodies in two individuals from South African rodent species, *A. namaquensis* and *T. leucogaster* by Immunofluorescence test using a panel of antigens from HTNV, PUUV and PHV respectively. Ours is the first known study to attempt the identification and isolation of hantaviruses in South Africa and Namibia.

In addition, a convenience serosurvey was also conducted; residual serum samples from NHLS laboratories at Tygerberg hospital were tested for the presence of hantavirus-specific IgG antibodies using an in-house ELISA based on recombinant hantavirus N antigens from two European hantaviruses, DOBV and PUUV. Of 161 human serum specimens tested, 11.2% were reactive against DOB-rN and 5.0% against PUU-rN, with 2.5% reactive against both recombinant antigens. These results from the ELISAs need to be confirmed by Western blot followed by serotyping by FRNT; however an indigenous virus will first have to be identified and isolated on cell culture before the latter can be attempted. Our study is the second known study conducted in South Africa to determine seroprevalence of hantavirus specific antibodies in the human population in the Western Cape and in South Africa. Lee *et al* (1999) found evidence of hantavirus in the general population as well as in patients hospitalised for acute renal failure, pulmonary oedema and glomerulonephritis.

In order to obtain a more representative and accurate picture of hantavirus seroprevalence in the human population, at least 1000 residual sera will be collected from laboratories in the Western Cape region in the next phase of the study and screened by ELISA; positive results will be confirmed with the more specific immunoblot assay.

It is important to conduct studies to investigate the occurrence of hantaviruses, even though no clinical case has been reported in South Africa, because it is through these studies that rodent populations can be surveyed so that society is equipped to respond more rapidly and diagnose accurately in the event of an outbreak. There is a possibility that hantavirus disease is occurring in southern Africa but is incorrectly diagnosed as either another viral haemorrhagic fever, leptospirosis, disease caused by a rodent-borne bacteria *Leptospira* sp. (Levett, 2001) or rickettsiosis caused by *Rickettsia africae* which is a tick-borne bacterium (Olano & Walker, 2009). The lack of state-of-the-art medical facilities and a case definition in South Africa and elsewhere on the continent may also contribute to the inability to diagnose hantavirus disease correctly in patients.

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Addendum A

Ethical approval – Committee for Experimental Animal Research (CEAR)



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4 March 2009

Miss NL Ithete
Division of Medical Virology

Dear Miss Ithete

RESEARCH PROJECT : "Molecular identification and characterisation of rodent and shrew borne hantaviruses."

PROJECT NUMBER : P09/01/002

The Committee for Experimental Animal Research considered your application for the approval of the abovementioned project on 25 February 2009. In principle the Committee is in agreement with the project, but requested that you should attend to the following matters:

1. Statements regarding the safety aspects of the experimental design must be added.
2. The BSL3 facility manager should also approve the protocol and it should be mentioned in the document.
3. Indicate under whose supervision the student will be trained in the procedures of BSL3 facility?
4. Adequate safety measures should be taken by the researchers when handling the animals e.g. masks should also be worn. This should be added on p.18 under the heading "Biohazard Statement".
5. Explain how animals will be restrained in the field for euthanization.
6. It should be stated that no trapping of animals in Namibia will take place before the appropriate permits are in place.
7. It should be indicated how many animals will be killed in total (p.15).

On receipt of the revised application form this project will be considered for final approval.

Yours faithfully

FRANKLIN WEBER
RESEARCH DEVELOPMENT AND SUPPORT (TYGERBERG)
Tel: +27 21 938 9657 / E-mail: fweb@sun.ac.za

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25 March 2009

Miss NL Ithete
Division of Medical Virology

Dear Miss Ithete

RESEARCH PROJECT : "Molecular identification and characterisation of rodent and shrew borne hantaviruses."

PROJECT NUMBER : P09/01/002

At a meeting of the Committee for Experimental Animal Research that was held on 25 February 2009 the above project was approved on condition that further information that was required, be submitted.

This information was supplied and the project was finally approved on 25 March 2009 **for a period of one year from this date**. This project is therefore now registered and you can proceed with the work. Please quote the above-mentioned project number in all further correspondence.

Please note that a progress report (obtainable on the website of our Division) should be submitted to the Committee before the year has expired. The Committee will then consider the continuation of the project for a further year (if necessary)

Yours faithfully

FRANKLIN WEBER
RESEARCH DEVELOPMENT AND SUPPORT (TYGERBERG)
Tel: +27 21 938 9657 / E-mail: fweb@sun.ac.za



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Addendum B

Ethical Approval – Human Research Ethics Committee



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27 February 2009 **MAILED**

Miss N Ithete
Department of Medical Virology
Stellenbosch University
PO Box 19063
Tygerberg
7505

Dear Miss Ithete

"Molecular Identification and Characterisation of Rodent and Shrew-borne Hantaviruses."

ETHICS REFERENCE NO: N09/02/058

RE : PROVISIONAL APPROVAL

It is my pleasure to inform you that the abovementioned project has been provisionally approved on 26 February 2009 for a period of one year from this date. You may start with the project, but this approval will however be submitted at the next meeting of the Committee for Human Research for ratification, after which we will contact you again.

Notwithstanding this approval, the Committee can request that work on this project be halted temporarily in anticipation of more information that they might deem necessary to make their final decision.

Please quote the abovementioned project number in all future correspondence.

Please note that a progress report (obtainable on the website of our Division) should be submitted to the Committee before the year has expired. The Committee will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly and subjected to an external audit.


Please note that in line with the recent changes to research ethics guidelines, including the Declaration of Helsinki, the CHR requires that all researchers specifically request and motivate for a "waiver of informed consent" for retrospective clinical audits.

Federal Wide Assurance Number: 00001372
Institutional Review Board (IRB) Number: IRB0005239


The Committee for Human Research complies with the SA National Health Act No.61 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 Part 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

27 February 2009 11:14

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Yours faithfully

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02 April 2009

MAILED

Miss N Ithete
Department of Medical Virology
Stellenbosch University
PO Box 19063
Tygerberg
7505

Dear Miss Ithete

"Molecular Identification and Characterisation of Rodent-and Shrew-borne Hantaviruses."

ETHICS REFERENCE NO: N09/02/058

RE : RATIFICATION

At a meeting that was held on 01 April 2009, the Committee for Human Research ratified the approval of the above project by the Chairperson.

Yours faithfully

MRS ELVIRA ROHLAND

RESEARCH DEVELOPMENT AND SUPPORT

Tel: 021 938 9677 / E-mail: elr@sun.ac.za

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02 April 2009 11:52

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Tel.: +27 21 938 9075 • Faks/Fax: +27 21 931 3352



UNIVERSITEIT • STELLENBOSCH • UNIVERSITY
jou kennisvennoot • your knowledge partner

07 May 2010

MAILED

Miss N Ithete
Department of Medical Virology
Stellenbosch University
PO Box 19063
Tygerberg
7505

Dear Miss Ithete

"Molecular Identification and Characterisation of Rodent-and Shrew-borne Hantaviruses."

ETHICS REFERENCE NO: N09/02/058

RE : PROGRESS REPORT

At a meeting of the Health Research Ethics Committee that was held on 21 April 2010, the progress report for the abovementioned project has been approved and the study has been granted an extension for a period of one year from this date.

Please note that to employ Virology registrars and clinicians from the renal and respiratory units would require an amendment to your protocol.

Please remember to submit progress reports in good time for annual renewal in the standard HREC format.

Approval Date: 21 April 2010

Expiry Date: 21 April 2011

Yours faithfully

MRS MERTRUDE DAVIDS

RESEARCH DEVELOPMENT AND SUPPORT

Tel: 021 938 9207 / E-mail: mertrude@sun.ac.za

Fax: 021 931 3352

07 May 2010 15:20

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Fakulteit Gesondheidswetenskappe · Faculty of Health Sciences



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23 June 2010

MAILED

Miss N Ithete
Department of Medical Virology
Stellenbosch University
PO Box 19063
Tygerberg
7505

Dear Miss Ithete

"Molecular Identification and Characterisation of Rodent-and Shrew-borne Hantaviruses."

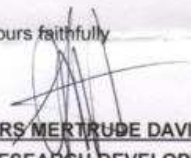
ETHICS REFERENCE NO: N09/02/058

RE : AMENDMENT

Your letter dated 3 June 2010 refers.

The Chairperson of the Health Research Ethics Committee approved the amended documentation in accordance with the authority given to him by the Committee.

Yours faithfully


MRS MERTRUDE DAVIDS

RESEARCH DEVELOPMENT AND SUPPORT

Tel: 021 938 9207 / E-mail: mertrude@sun.ac.za

Fax: 021 931 3352

23 June 2010 14:02

Page 1 of 1



Fakulteit Gesondheidswetenskappe · Faculty of Health Sciences

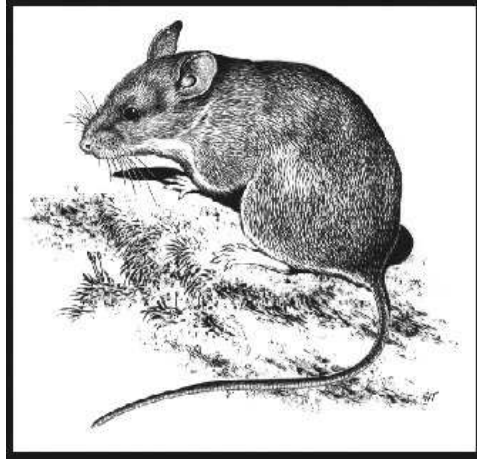


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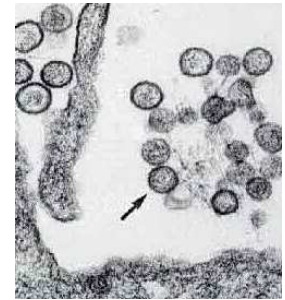
A. Information and consent form for the recruitment of patients

Research project: Do hantaviruses occur in Southern Africa?

Hantaviruses are members of the *Bunyaviridae* family. Their natural hosts are rodents and shrews. So far, more than 30 different hantavirus species have been found infecting different species of rodent or shrew.



Some types of hantaviruses may cause zoonotic disease in human beings. Humans are typically infected by inhaling aerosolized excreta of infected rodents, e.g. when cleaning rodent-infested buildings. The two recognised hantavirus-associated human disease entities are haemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (HCPS).



Until recently hantaviruses were known to occur in Asia, Europe and the Americas. However, in 2006 the first African hantavirus was identified in and isolated from the African wood mouse (*Hylomyscus simus*) in Guinea, West Africa. The new virus was named Sangassou, after the area where it was first detected. Soon afterwards genetic evidence for another hantavirus was detected in a Theresé's shrew (*Crocidura theresae*), also in West

Africa, and named Tanganya virus.

The identification of these two African hantaviruses has highlighted the possibility that there may be yet other hantaviruses in Africa that are yet to be identified. These viruses might infect and cause disease in human beings. It is possible that infections and diseases caused by yet unknown hantaviruses may be confused with other severe diseases such as rickettsiosis, severe pneumonia, leptospirosis, viral haemorrhagic fevers etc. Without specific laboratory testing, a hantavirus aetiology may not be recognised (as was the case with HFRS in Eurasia and HCPS in America before the responsible viruses were discovered).

Our study aims to identify hantaviruses and possible hantavirus diseases in southern Africa. So far, the occurrence of these viruses and any possibly related illnesses is not known from our region. The study has been approved by the ethics committee of the University of Stellenbosch. We would like to test patients with clinical disease that might possibly be due to hantavirus infection, to see whether hantavirus infections do occur at all and if so, whether they may be causing disease. If we find evidence of hantavirus infection in patients a clinical case definition and diagnostic assays can be developed to help diagnose future cases.

We are looking for patients with clinical symptoms that might be indicative of hantavirus disease. These symptoms are:

- **Fever of unknown origin**, not responding to antibiotic treatment,

PLUS

- any of the following, of unknown aetiology: **acute renal failure, acute respiratory failure, acute liver failure, pneumonia and/or mucosal bleeding.**

We need serum samples from patients fulfilling this case definition. The samples will be tested for the presence of hantavirus antibodies by an in-house ELISA assay. A patient information sheet and consent form have been developed and approved. A study sample form (asking for a minimum set of clinical information) is also available.

For more information on the project please contact:

Miss Ndapewa Ithete
Division of Medical Virology
021 938 9360
14567008@sun.ac.za

Prof Wolfgang Preiser
Division of Medical Virology
021 938 9353
preiser@sun.ac.za

PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM

TITLE OF THE RESEARCH PROJECT: MOLECULAR IDENTIFICATION AND CHARACTERISATION OF RODENT- AND SHREW-BORNE HANTAVIRUSES

REFERENCE NUMBER: **N02/09/058**

PRINCIPAL INVESTIGATOR: **Ndapewa Ithete**

ADDRESS: **Division of Medical Virology, Department of Pathology, Faculty of Health Sciences, Stellenbosch University.**

CONTACT NUMBER: **021 938 9360**

You are being invited to take part in a research project. Please take some time to read the information presented here, which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how you could be involved. Also, your participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do agree to take part.

This study has been approved by the **Committee for Human Research at Stellenbosch University** and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.

What is this research study all about?

This study aims to identify hantaviruses and hantavirus diseases in southern Africa. So far, the occurrence of these viruses and any possibly related illnesses is not known from our region. The idea is to test patients with clinical disease that might possibly be due to hantavirus infection for the evidence of this, to see whether hantavirus infections do occur at all and if so, whether they may be causing disease.

Patients will be recruited at two hospitals: Tygerberg Hospital and Groote Schuur Hospital in Cape Town.

Blood samples from patients with clinical symptoms that might be indicative of hantavirus disease will be collected. Such symptoms include fever of unknown origin not responding to antibiotic treatment, plus any of the following: acute renal failure, acute respiratory failure, and acute liver failure, mucosal bleeding, all of unknown aetiology. Blood samples will be obtained at different time points after onset of disease, frozen and later tested for hantaviruses. In addition, if and when biopsies (e.g. renal biopsies in cases of acute renal failure) are performed for routine diagnostic purposes, residual sample material will also be obtained for hantavirus testing.

The aim of this project is to identify and characterise novel hantaviruses in patients whose clinical illness matches that seen in patients with proven hantavirus infection in other parts of the world. Patients will be selected based on clinical criteria and patient samples will be tested by molecular and serological methods. If we find evidence of hantavirus infection in such patients this will help us to define a clinical case definition and to develop diagnostic assays that might in future help to diagnose hantavirus-infected patients.

When routine blood samples are taken from the participating patients, another (extra) tube of blood will be collected for the purposes of this study. This specimen will then be tested for the presence of anti-hantavirus antibodies and of hantavirus genome.

Why have you been invited to participate?

You have been chosen to participate because you are currently suffering from a disease which resembles that caused by hantaviruses in other parts of the world.

What will your responsibilities be?

To provide us with an extra blood sample when blood is taken for routine purposes anyway. One tube with 10 ml (2 to 3 teaspoons) of blood will be collected.

Will you benefit from taking part in this research?

You will not benefit from this research directly yourself. Your participation would however be very valuable as it might lead to the identification of new viruses. This contributes to medical knowledge in general and will allow us to develop a clinical case definition of hantavirus disease in South Africa.

Possible identification of hantaviruses will allow the development of diagnostic assays to be used to determine whether future patients have hantavirus infection.

Are there in risks involved in your taking part in this research?

There is minimal risk involved in your participation in this study as the extra blood specimen to be taken will be obtained at the same time as one or more for routine diagnostic testing (no extra needle prick involved).

If you do not agree to take part, what alternatives do you have?

Whether you wish to take part in this study or not will not affect your clinical care in any way.

Who will have access to your medical records?

All personal information collected will be treated as confidential and access to it will be strictly controlled and limited to the investigators. All identifying information will be anonymised at the earliest possible time point. All patient specimens will be assigned numbers for identification purposes when used in a publication or thesis.

What will happen in the unlikely event of some form injury occurring as a direct result of your taking part in this research study?

This is not applicable, as the only inconvenience is drawing an extra tube of blood from you at the same time as samples are being obtained for routine purposes anyway.

Will you be paid to take part in this study and are there any costs involved?

You will not be paid to take part in the study, and you will not incur any costs either.

Is there any thing else that you should know or do?

- You can contact Ms Ndapewa lthete at telephone 021 938 9360 if you have any further queries or encounter any problems.
- You can contact the Committee for Human Research at 021-938 9207 if you have any concerns or complaints that have not been adequately addressed by your study doctor.
- You will receive a copy of this information and consent form for your own records.

Declaration by participant

By signing below, I agree to take part in a research study entitled **Molecular Identification and Characterisation of Rodent- and Shrew-borne Hantaviruses.**

I declare that:

- I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is **voluntary** and I have not been pressurised to take part.
- I may choose to leave the study at any time and will not be penalised or prejudiced in any way.

- I may be asked to leave the study before it has finished, if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan, as agreed to.

Signed at (*place*) on (*date*) 2010.

.....
Signature of participant

.....
Signature of witness

5.4.1 Declaration by investigator

I (*name*) declare that:

- I explained the information in this document to
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research, as discussed above
- I did/did not use a interpreter. (*If a interpreter is used then the interpreter must sign the declaration below.*)

Signed at (*place*) on (*date*) 2010.

.....
Signature of investigator

.....
Signature of witness

5.5 Declaration by interpreter (if applicable)

I (*name*) declare that:

- I assisted the investigator (*name*) to explain the information in this document to (*name of participant*) using the language medium of Afrikaans/Xhosa (delete as applicable).
- We encouraged him/her to ask questions and took adequate time to answer them.
- I conveyed a factually correct version of what was related to me.
- I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her question satisfactorily answered.

Signed at (*place*) on (*date*) 2010

.....
Signature of interpreter

.....
Signature of witness

B. Information pamphlet for the anonymous serosurvey

Anonymous serosurvey: Do human hantavirus infections occur in South Africa?

Aim:

- This unlinked anonymous serosurvey aims to identify the occurrence of hantaviruses infecting human beings in southern Africa.

Design:

- Convenience collection ("opportunistic" samples): using residual serum (clotted blood) samples from human patients sent for routine laboratory testing that are left over after the requested tests have been done and would otherwise be discarded.
- Serum samples originating from NHLS laboratories situated in rural areas of the Western Cape or receiving referrals from such areas.
- Samples are anonymised upon receipt; only information on date of collection, age, sex and source laboratory is retained but no demographic or medical information on the patient.
- Ethics approval will be sought from the Health Research Ethics Committee of the Faculty of Health Sciences, University of Stellenbosch.

What we are looking for:

- Colleagues from NHLS laboratories in the Western Cape who are willing to participate by sending us residual (left-over) serum samples that would otherwise be discarded.
- Important: Only serum samples are required (blood containing additives such as EDTA or heparin will not be suitable for the serological methods to be used).
- The samples should have been obtained and handled according to standard clinical and laboratory practices and stored in a refrigerator (at approximately +4 °C).
- Residual samples should be at least 0.3 ml.
- In some other countries the average seroprevalence is around 1%. Based on this, between 1500 and 3000 specimens (but not all necessarily from the same area) would be desirable whilst still being a practicable quantity.
- Upon receipt at Tygerberg, the remaining serum will be transferred into a new vial by the research team. If patient identifiers are present on the sample tube, only age and sex will be recorded and all original sample tubes will be discarded after the serum has been transferred.
- Sera will be tested for the presence of hantavirus-specific antibodies by means of a cascade of antibody tests, starting with screening assays likely to yield a high number of non-specific reactive results which are then confirmed by using more specific assays such as microneutralisation.

Rationale:

- This approach is similar to (but more focussed and with a smaller geographical coverage than) that used in the United Kingdom for the Health Protection Agency's (HPA) Seroepidemiology Programme (cf. <http://www.hpa.org.uk/ProductsServices/InfectiousDiseases/ServicesActivities/SeroepidemiologyProgramme>): "Sera used are residues of specimens submitted for diagnostic testing, ... are anonymised (retaining age, sex, date of collection and source laboratory only). Collection of

sera is continuing through collaboration with ... laboratories throughout England and Wales."

- Like the HPA Seroepidemiology Programme, this study "is focused on cross-sectional antibody prevalence ... to help in the understanding of the epidemiology and burden of infectious diseases of public health importance, and how this may be changing. This provides key evidence to assist with making informed decisions regarding health policy where intervention is possible." A recent example of a research study in which this resource was used is Miller *et al.*, Incidence of 2009 pandemic influenza A H1N1 infection in England: a cross-sectional serological study. Lancet. 2010 Mar 27;375(9720):1100-8.

Limitations:

- Limited information will be collected on patient demographics (including occupation, location of possible exposure) or medical condition.
- Given that the samples will originate from patients who have laboratory tests done within the public health sector, and given that such testing in South Africa is done less easily and less frequently than in most developed countries, the collection is likely to compromise predominantly patients with clinical illness and may therefore overestimate true population prevalence rates.
- However the purpose of this anonymous serosurvey is to ascertain whether hantavirus-specific antibodies occur at all in human populations in the Western Cape, as a marker of past exposure to hantaviruses, and not to link hantavirus infection and possible hantavirus disease (this is addressed in a separate, ongoing study at Groote Schuur and Tygerberg hospitals, aiming to test patients with acute clinical disease that might possibly be due to hantavirus infection).

For more information on the project please contact:

Miss Ndapewa Ithete

021 938 9360

14567008@sun.ac.za

Prof Wolfgang Preiser

021 938 9353

preiser@sun.ac.za

Division of Medical Virology, Faculty of Health Sciences, Tygerberg

Addendum C

Rodent and shrew trapping locations

Beaufort West

close to human dwellings

Rhabdomys pumilio

BwRp 1
BwRp 2
BwRp 3
BwRp 4
BwRp 5
BwRp 6
BwRp 7
BwRp 8
BwRp 9
BwRp 10
BwRp 11
BwRp 12
BwRp 13
BwRp 14
BwRp 15
BwRp 16
BwRp 17
BwRp 20
BwRp 21
BwRp 22
BwRp 23
BwRp 24
BwRp 25
BwRp 26
BwRp 27
BwRp 28
BwRp 29
BwRp 30
BwRp 31
BwRp 32
BwRp 33

Elandskloof

Farm

Rhabdomys pumilio

EkRp 1
EkRp 2
EkRp 3
EkRp 4
EkRp 5
EkRp 6
EkRp 7
EkRp 8
EkRp 9
EkRp 10
EkRp 11
EkRp 12
EkRp 13
EkRp 14
EkRp 15
EkRp 16
EkRp 17
EkRp 18
EkRp 19
EkRp 20
EkRp 21
EkRp 22
EkRp 23
EkRp 24
EkRp 25
EkRp 26
EkRp 27
EkRp 28
EkRp 29
EkRp 30

Goegap, Springbok

old sheep farm

Rhabdomys pumilio

GpRp M 1	GpRp M 22	GpRp O 19	GpRp A 12
GpRp M 2	GpRp M 23	GpRp O 20	GpRp A 13
GpRp M 3	GpRp M 24	GpRp O 21	GpRp A 14
GpRp M 4	GpRp M 25	GpRp O 22	GpRp A 15
GpRp M 5	GpRp M 26	GpRp O 23	GpRp A 16
GpRp M 6	GpRp M 27	GpRp O 24	GpRp A 17
GpRp M 7	GpRp M 28	GpRp O 25	GpRp A 18
GpRp M 8	GpRp M 29	GpRp O 26	GpRp A 19
GpRp M 9	GpRp M 30	GpRp O 27	GpRp A 20
GpRp M 10	GpRp M 31	GpRp O 28	GpRp A 21
GpRp M 11	GpRp O 7	GpRp A 1	GpRp A 22
GpRp M 12	GpRp O 8	GpRp A 2	GpRp A 23
GpRp M 13	GpRp O 9	GpRp A 3	GpRp A 24
GpRp M 14	GpRp O 10	GpRp A 4	GpRp A 25
GpRp M 15	GpRp O 11	GpRp A 5	GpRp A 26
GpRp M 16	GpRp O 12	GpRp A 6	GpRp A 27
GpRp M 17	GpRp O 14	GpRp A 7	GpRp A 28
GpRp M 18	GpRp O 15	GpRp A 8	GpRp A 29
GpRp M 19	GpRp O 16	GpRp A 9	GpRp A 30
GpRp M 20	GpRp O 17	GpRp A 10	GpRp A 31
GpRp M 21	GpRp O 18	GpRp A 11	

Saccostomys sp

Gp Sp 6

Mus musculus

GR. m 1

GR. m 2

GR. m 3

GR. m 4

GR. m 5

Parotomys sp

GR f 1

**Jonkershoek,
Stellenbosch**

Fragmented habitat Nature
reserve

Rhabdomys pumilio

J.Hoek Rp 1	JHRp 20
J.Hoek Rp 2	JHRp 21
J.Hoek Rp 3	JHRp 23
J.Hoek Rp 4	JHRp 24
J.Hoek Rp 5	JHRp 26
J.Hoek Rp 6	JHRp 27
JHRp 1	JHRp 28
JHRp 2	JHRp 29
JHRp 3	JHRp 30
JHRp 4	JHRp *31
JHRp 5	JHRp *32
JHRp 6	JHRp *33
JHRp 7	JHRp *34
JHRp 8	JHRp *35
JHRp 12	JHRp *36
JHRp 17	JHRp *37
JHRp 18	JHRp *38
JHRp 19	JHRp *39

Otomys irroratus

VET O. irr 1
VET O. irr 2

Shrews

J. Hoek Shrew 1
J. Hoek Shrew 2
J. Hoek Shrew 3

Laingsburg

Old sheep
farm

Rhabdomys pumilio

LBRp 1
LBRp 2
LBRp 3
LBRp 4
LBRp 5
LBRp 6
LBRp 7
LBRp 8
LBRp 9
LBRp 10
LBRp 11
LBRp 12
LBRp 13
LBRp 14
LBRp 15
LBRp 17
LBRp 18
LBRp 20
LBRp 22
LBRp 23
LBRp 24

Aethomys

sp.

Ae LB 1
Ae LB 2

Otomys unisulcatus

Ou LB 1
Ou LB 2
Ou LB 3

shrew

shrew LB 1

Loeriesfontein

farm

Rhabdomys pumilio

LfRp 1

LfRp 2

LfRp 3

LfRp 4

LfRp 5

LfRp 6

LfRp 7

LfRp 8

LfRp 9

LfRp 10

LfRp 11

LfRp 12

LfRp 14

Middelvlei, Stellenbosch

Fragmented habitat ,
farming

Rhabdomys pumilio

MvRp 1
MvRp 2
MvRp 3
MvRp 4
MvRp 5
MvRp 6
MvRp 7
MvRp 8
MvRp 9
MvRp 10
MvRp 11
MvRp 12
MvRp 13
MvRp 14
MvRp 15
MvRp 16
MvRp 17
MvRp 18
MvRp 19
MvRp 20
MvRp 21
MvRp 22
MvRp 23
MvRp 24
MvRp 25
MvRp 26
MvRp 28
MvRp 29
MvRp 30
MvRp 31

**Mulderbosch,
Stellenbosch**

fragmented habitat,
farming

Rhabdomys pumilio

MBA Rp 1	MB Rp 7	MBM Rp 6
MBA Rp 2	MB Rp 8	MBM Rp 7
MBA Rp 3	MB Rp 9	MBM Rp 8
MBA Rp 4	MB Rp 10	MBM Rp 9
MBA Rp 5	MB Rp 11	MBM Rp 10
MBA Rp 6	MB Rp 12	MBM Rp 11
MBA Rp 7	MB Rp 13	MBM Rp 12

MBA Rp 8	MB Rp 14	MBM Rp 13
MBA Rp 9	MB Rp 15	MBM Rp 14
MBA Rp 10	MB Rp 16	MBM Rp 15
MBA Rp 11	MB Rp 17	MBM Rp 16
MBA Rp 12	MB Rp 18	MBM Rp 17
MBA Rp 13	MB Rp 19	MBM Rp 18
MBA Rp 14	MB Rp 20	MBM Rp 19
MBA Rp 15	MB Rp 21	MBM Rp 20
MBA Rp 16	MB Rp 22	MBM Rp 21
MBA Rp 17	MB Rp 23	MBM Rp 22
MBA Rp 18	MB Rp 24	MBM Rp 23
MBA Rp 19	MB Rp 25	MBM Rp 24
MBA Rp 20	MB Rp 26	MBM Rp 25
MBA Rp 22	MB Rp 27	MBM Rp 26
MBA Rp 25	MB Rp 28	MBM Rp 27
MBA Rp 26	MB Rp 29	MBM Rp 28
MBA Rp 27	MB Rp 30	MBM Rp 29
MBA Rp 29	MB Rp 31	MBM Rp 24
MBA Rp 30	MB Rp 32	MBM Rp 25
MB Rp 1	MB Rp 33	MBM Rp 26
MB Rp 2	MBM Rp 1	MBM Rp 27
MB Rp 3	MBM Rp 2	MBM Rp 28
MB Rp 4	MBM Rp 3	MBM Rp 29
MB Rp 5	MBM Rp 4	MBM Rp 30
MB Rp 6	MBM Rp 5	MBM Rp 31

shrew

MB shrew 1

Otomys
unisulcatus

MB O.irr 1
MB O.irr 2
MB O.irr 3
MB O.irr 4

Riverlands

Shrew

Riverlands shrew 1

Stellenbosch

urban

Rattus rattus

RAT 1

RAT 2

RAT 3

RAT 4

RAT 5

RAT 6

RAT 7

RAT 8

RAT 9

RAT 10

RAT 11

RAT 12

RAT 13

Sutherland
sheep farm

Rhabdomys pumilio

SIRp 1
SIRp 2
SIRp 3
SIRp 4
SIRp 5
SIRp 6
SIRp 7
SIRp 8
SIRp 9
SIRp 10
SIRp 11
SIRp 12
SIRp 13

Aethomys

sp.

AeSI 1
AeSI 2
AeSI 3
AeSI 4

Gerbilluris paeba

GpSI 1
GpSI 2
GpSI 3
GpSI 4
GpSI 5
GpSI 6
GpSI 7
GpSI 8
GpSI 9

Otomys unisulcatus

OuSI 1
OuSI 2
OuSI 3

Shrew

Shrew SI 1

Vergelegen

Wine farm

Rhabdomys pumilio

Vg/Vo Rp 1

Vg/Vo Rp 2

Vg/Vo Rp 3

Vg/Vo Rp 4

Vg/Vo Rp 5

Vg/Vo Rp 6

Vg/Vo Rp 7

Vg/Vo Rp 8

Vg/Vo Rp 9

Vg/Vo Rp 10

Vg/Vo Rp

10#

Vg/Vo Rp 12

Vg/Vo Rp 13

Vg/Vo Rp 14

Vg/Vo Rp 15

Vg/Vo Rp

17#

Vg/Vo Rp 17

Vg/Vo Rp 18

Vg/Vo Rp 20

Vg Rp 21

Vg Rp 22

Vg Rp 23

Vg Rp 24

Voelvodam

Nature reserve

Rhabdomys pumilio

VdRp 24

VdRp 26

VdRp 27

VdRp 30

Waterval farm

farm

Rhabdomys

pumilio

WfRp 1
WfRp 2
WfRp 3
WfRp 4
WfRp 5
WfRp 6
WfRp 7
WfRp 8
WfRp 9
WfRp 10
WfRp 11
WfRp 12
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WfRp 24
WfRp 25
WfRp 26

Keetmanshoop (Namibia)

Shrew

keetmans shrew 1

Addendum D

Convenience sero-survey specimens

A. List of sera from female patients

Specimen number	Age	Sex	Specimen number	Age	Sex
HAN-5	37	F	HAN-77	62	F
HAN-6	75	F	HAN-78	-	F
HAN-11	53	F	HAN-79	31	F
HAN-13	83	F	HAN-80	54	F
HAN-14	3	F	HAN-81	37	F
HAN-15	74	F	HAN-82	43	F
HAN-16	68	F	HAN-84	54	F
HAN-17	1	F	HAN-87	34	F
HAN-18	67	F	HAN-88	45	F
HAN-19	16	F	HAN-89	48	F
HAN-21	32	F	HAN-93	28	F
HAN-23	27	F	HAN-97	19	F
HAN-24	72	F	HAN-98	46	F
HAN-26	47	F	HAN-99	50	F
HAN-27	31	F	HAN-100	45	F
HAN-29	37	F	HAN-106	15	F
HAN-30	31	F	HAN-108	32	F
HAN-32	80	F	HAN-109	30	F
HAN-33	52	F	HAN-112	43	F
HAN-35	66	F	HAN-113	51	F
HAN-36	59	F	HAN-114	32	F
HAN-37	41	F	HAN-115	28	F
HAN-39	47	F	HAN-117	72	F
HAN-40	37	F	HAN-118	24	F
HAN-41	16	F	HAN-119	38	F
HAN-42	20	F	HAN-120	88	F
HAN-44	86	F	HAN-121	38	F
HAN-48	86	F	HAN-124	24	F
HAN-49	76	F	HAN-125	76	F
HAN-50	54	F	HAN-127	67	F
HAN-51	59	F	HAN-128	45	F
HAN-52	27	F	HAN-129	19	F
HAN-54	48	F	HAN-131	29	F
HAN-55	30	F	HAN-132	34	F
HAN-56	60	F	HAN-133	69	F
HAN-57	-	F	HAN-137	74	F
HAN-58	83	F	HAN-138	74	F
HAN-59	65	F	HAN-139	38	F
HAN-61	42	F	HAN-141	63	F
HAN-62	52	F	HAN-142	33	F
HAN-63	39	F	HAN-145	46	F
HAN-64	83	F	HAN-146	37	F
HAN-65	56	F	HAN-147	37	F
HAN-67	27	F	HAN-148	80	F
HAN-68	51	F	HAN-152	29	F
HAN-70	59	F	HAN-154	18	F
HAN-72	27	F	HAN-156	10	F
HAN-73	26	F	HAN-157	49	F
HAN-74	46	F	HAN-159	29	F
HAN-76	70	F			

List of sera from male patients

Specimen number	Age	Sex	Specimen number	Age	Sex
HAN-1	68	M	HAN-94	60	M
HAN-2	48	M	HAN-95	22	M
HAN-3	31	M	HAN-96	61	M
HAN-4	41	M	HAN-101	45	M
HAN-7	50	M	HAN-102	28	M
HAN-8	21	M	HAN-103	58	M
HAN-9	40	M	HAN-104	46	M
HAN-10	55	M	HAN-105	20	M
HAN-12	61	M	HAN-107	34	M
HAN-20	45	M	HAN-110	59	M
HAN-22	56	M	HAN-111	46	M
HAN-25	62	M	HAN-116	58	M
HAN-28	73	M	HAN-122	28	M
HAN-31	33	M	HAN-123	62	M
HAN-34	51	M	HAN-126	28	M
HAN-38	54	M	HAN-130	16	M
HAN-43	15	M	HAN-134	24	M
HAN-45	82	M	HAN-135	59	M
HAN-46	57	M	HAN-136	-	M
HAN-47	13	M	HAN-140	41	M
HAN-53	42	M	HAN-143	59	M
HAN-60	47	M	HAN-144	61	M
HAN-66	52	M	HAN-149	49	M
HAN-71	68	M	HAN-150	46	M
HAN-75	81	M	HAN-151	68	M
HAN-83	51	M	HAN-153	40	M
HAN-85	20	M	HAN-155	13	M
HAN-86	65	M	HAN-158	16	M
HAN-90	24	M	HAN-160	34	M
HAN-91	50	M	HAN-161	71	M
HAN-92	70	M			